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TITLE OF THE INVENTION (500 characters max)

Pili in Mycobacterium tuberculosis

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[Page 1 of 2]

Respectfully submitted

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U.S. Provisional Patent Application

TITLE: Pili in *Mycobacterium tuberculosis*

INVENTORS: Richard Friedman, Jorge Diron & Christopher Alteri

FILED: March 1, 2004

Description.

Background. *Mycobacterium tuberculosis* is the bacterial agent responsible for human pulmonary tuberculosis. Almost one third of the world's population suffers from this infectious disease. The *M. tuberculosis* bacillus is highly infectious and is spread by aerosols from infected individuals with active pulmonary disease. Over three million people die yearly from tuberculosis, the largest single infectious cause of mortality worldwide. Tuberculosis is still a persistent health problem in the U.S.A. due in part to the human immunodeficiency virus (AIDS) epidemic. AIDS patients are highly susceptible to infection with *M. tuberculosis* and other non-tuberculosis mycobacteria that seldom infect individuals with intact immune systems. For many bacterial pathogens, the ability to produce proteinaceous adhesins in the form of hair-like structures, called pili, are an important pathogenic attribute since they mediate close interaction and colonization with host cells. However, in the case *M. tuberculosis* no pili structures have ever been observed and it is still an enigma as to whether pili may play a role in human tuberculosis infection.

We have, for the first time, identified and partially characterized pili-like structures on *M. tuberculosis* strains as 2-5-nm-wide fibers that associate into a highly hydrophobic meshwork of variable dimensions. These structures, herein called *M. tuberculosis* pili (or Mtp), are produced *in vitro* and were demonstrated to react with antibodies present in human convalescent sera obtained from tuberculosis patients. These novel findings are the basis for the present patent disclosure, and may have important implications in terms of immunoprophylaxis, prevention and diagnosis of this historically deadly disease.

Molecular Mechanisms of *M. tuberculosis* Pathogenesis. Although tuberculosis is now recognized as a major public health problem nationally and internationally, there is a need for more information on the basic molecular mechanisms of *M. tuberculosis* pathogenesis and the mechanisms of drug resistance and immunity to this pathogen. Key to tuberculosis pathogenesis is the ability of the bacilli to adhere and enter macrophages and possibly other host cell types, to resist killing, and to replicate in these intracellular sites. The specific molecular mechanisms *M. tuberculosis* uses in these processes are unknown. But the recent DNA sequencing and annotation of the laboratory strain *M. tuberculosis* H37Rv and the clinical isolate CDC1551 genomes have added much to our general knowledge of the genetics of this microbial pathogen.

Bacterial Adherence. Adherence to host tissues is an essential and complex first stage for bacterial colonization for the establishment of bacterial infectious disease. In many cases, adherence is mediated by one or more adhesins that can act simultaneously or in distinct steps of an infectious process. Adhesins, in the form of pili or outer membranes proteins, may mediate direct or indirect binding to host cells. A great deal of information is available in terms of the interaction and trafficking of *M. tuberculosis* within macrophages of the immune system. It is reasonable to presume that the bacteria are able to express surface molecules devoted to the specific recognition of unique or common receptor components present on target tissues. Nevertheless, the mechanisms underlying the adherence properties of *M. tuberculosis* to the first line of epithelial cells before interacting with professional phagocytes are just beginning to be unraveled. Analysis of the genome sequence of *M. tuberculosis* has revealed various genes coding for putative adhesins and invasins, yet no fimbrial adhesins have been described in *M. tuberculosis*. In consequence, it is still unknown if these organisms colonize the respiratory epithelium of their human hosts prior to macrophage interaction in the alveolus.

SUPPORTING DATA

Data presented below show that *M. tuberculosis* is able to produce surface appendages resembling pili. The criteria for calling these structures pili are: 1) their morphology and dimensions are similar to those of the pili of other bacteria, 2) using standard methods to

isolate pili from other microorganisms, we have been successful in purifying these pili structures from *M. tuberculosis* and 3) Mtp binds to extracellular matrix proteins (ECM). These observations may open a new avenue to further understand the pathogenic mechanisms of this deadly human pathogen.

1. ***Mycobacterium tuberculosis* produces pili structures.** In the course of ultrastructural studies of pathogenic and attenuated strains of *M. tuberculosis* by negative staining and transmission electron microscopy (TEM), we noted the presence of fibrillar structures resembling pili (also called fimbriae) when the bacteria were propagated under suitable laboratory growth conditions. Namely, cultures of various *M. tuberculosis* strains (avirulent H37Ra, virulent H37Rv and CDC1551) were grown on 7H11 agar plates containing OADC for three weeks at 37°C. All work with virulent *M. tuberculosis* strains was done in the BSL-3 laboratory of Dr. Friedman. Plate grown bacteria were gently suspended in 4% formaldehyde and incubated overnight in a microfuge tube. The bacteria were negatively stained with 1% phosphotungstic acid (pH 7.4) on Formvar-coated copper grids and then observed in a Phillips CM12 electron microscope at 80 kV. All of the *M. tuberculosis* strains analyzed produced thin (2-5 nm-wide), aggregative, flexible hair-like appendages that protruded several microns away from the bacterial cell surface (**Fig. 1A, B and C**). The fine fibrillar structures, **herein called *M. tuberculosis* pili or Mtp**, tended to aggregate to each other forming a meshwork of variable dimensions that appeared associated with the bacteria or free in the supernatants. As a particular note, these fibrillar structures are morphologically reminiscent of the well-characterized curli structures produced by some enteric bacterial pathogens. Under these growth conditions, ~5% of the bacterial cells present in the culture samples analyzed by TEM possessed pili. Studies were done to confirm that the pili structures observed were bacterial in nature and not artifacts present in the bacterial growth media employed. We prepared electron microscopy grids with liquid medium or with water sitting on solid agar medium that had been incubated for the same time as inoculated cultures. **In these control studies we did not observe any fibrillar structures, indicating that the fibers seen in *M. tuberculosis* cultures were of bacterial origin.**

We were then interested in studying the effect of culture conditions on the production of pili by H37Ra, H37Rv, and CDC1551. To this aim, we used a panel of liquid and solid media (**Table 1**) for bacterial growth and qualitatively determined the level of pili production by negative staining and TEM. *M. tuberculosis* strains were plated as a lawn and grown for a period of 3 weeks at 37°C in a 5% CO_2 atmosphere. *M. tuberculosis* broth cultures in 7H9 were grown for 2 to 3 weeks and GAS broth cultures were grown for 5 weeks until both reached an OD_{650} of 1.50. The data summarized in Table 1 indicates that Mtp are likely controlled by environmental stimuli since the level of pili production varied depending on the growth media used. Further, the qualitative analysis demonstrates that the avirulent strain of *M. tuberculosis*, H37Ra, has a diminished capacity to produce pili as compared to H37Rv and CDC1551. Strikingly, the greatest difference between virulent and attenuated *M. tuberculosis* was observed in broth grown cultures, where *M. tuberculosis* H37Ra produces nearly undetectable levels of pili, while *M. tuberculosis* H37Rv and CDC1551, the clinical isolate, produce the highest level of pili in the culture conditions tested. Ten to 25% of *M. tuberculosis* H37Rv and CDC1551 grown in either 7H9 or GAS broth contained pili as observed by TEM.

In conclusion, we have for the first time shown that *M. tuberculosis* produces pili structures and their production is under the control of environmental growth conditions.

2. **Production of pili by other *Mycobacterium* species.** We extended the TEM analysis to other *Mycobacterium* species, including *M. bovis* BCG, *M. fortuitum* (a clinical isolate), and *M. smegmatis* strain 1-2c to determine the production of pili after growth under different culture conditions as done with *M. tuberculosis*. *M. bovis* BCG was grown as a lawn on 7H11 agar plates containing OADC for three weeks at 37°C, while *M. fortuitum* was grown in 7H9 broth containing glucose and Tween-80. *M. smegmatis* was grown in 7H9 broth

cultures with and without Tween-80, LB broth, on 7H10 agar plates with either glucose or glycerol, sheep blood agar, RPMMA agar, or on LB agar plates.

Table 1. *M. tuberculosis* pili production on various media as observed by TEM[1]

Strain	7H11 agar + OADC	3% Sheep Blood Agar	7H10 agar + glycerol	7H11 agar + glycerol	7H10 agar + glucose	RPMMA Agar***	7H9 broth + OADC + Tw*	GAS** broth + Tw*
H37Ra	+	++	+	++	+	-	-	-
H37Rv	+	++	+	+[2]	+[2]	-	+++	++
CDC1551	++	++	+[2]	+[2]	+[2]	+	+++	++

*[1] All plate grown cultures in the table were incubated in a 5% CO_2 atmosphere, [2] Very limited growth was observed in these conditions. * Tween 80 (Tw). ** glycerol-alanine salts (GAS) medium (58). *** RPMMA (reduced phosphate modified minimal A) agar is a defined minimal media on which mycobacteria can be grown (personal communication, James Megehee. +++ 10 to 25% of M. tuberculosis contain pili, ++ 5 to 10% bacteria contain pili, + less than 5% bacteria contain pili, - undetectable levels of pili.*

We noted that all of these species were able to produce thin, flexible, pili when grown at 37°C (**Fig. 1D, E and F**). Interestingly, *M. smegmatis* produced long, semi-flexible pili structures that tended to aggregate laterally and form bundles or rope-like structures (**Fig 1E**). This is in contrast to *M. tuberculosis* strains that produced curli-like pili (**Fig 1A-C**). *M. smegmatis* produced pili regardless of the growth temperature (either at 25° or 37°C) and the composition of the media employed. However, the level of pili produced by *M smegmatis* was significantly higher in 7H9 broth supplemented with glucose, with or without Tween-80, and in Luria-Bertani broth. The *M. smegmatis* pili were also produced on solid media, albeit to a lesser extent than in liquid media. Growth in a 5% CO_2 atmosphere on agar plates did not stimulate increased production of pili by *M. smegmatis*. It is apparent that environmental cues are regulating the production of pili in *M. smegmatis* as was observed in *M. tuberculosis*. In conclusion, production of pili appears to be a generalized phenomenon in *Mycobacterium* species.

3. **Identification and purification of *M. tuberculosis* pili (Mtp).** Our next goal was to purify and identify the nature of the pili structures observed in *M. tuberculosis* cultures. For safety reasons and ease of working under non-BSL-3 conditions, *M. tuberculosis* H37Ra was used for pili purification. H37Ra was grown at 37°C under a 5% CO_2 atmosphere for three weeks on one hundred 7H11 agar plates supplemented with OADC (**Table 1**). Cultures were Gram stained and acid-fast stained to confirm purity of the bacterial preparations. The heavy bacterial lawn obtained was harvested from the plates into 150 mM mono-ethanolamine buffer (pH 10.5) and the pili were mechanically sheared from the surface of the bacteria. The bacteria were separated by repeated low speed centrifugation and the supernatant containing pili was extracted with 2:1 choloroform:methanol to remove vesicular material. The upper aqueous phase and interphase that contained pili fibers was recovered. This fraction was centrifuged at 18,000 x *g* to completely remove bacteria, bacterial membranes and debris. The supernatant fraction containing the pili was then recovered and concentrated by ultracentrifugation for several hours at 4°C. The pelleted pili was resuspended in PBS and dialyzed to remove any salts. This preparation was further analyzed by TEM, which revealed the presence of abundant pili aggregates (**Fig. 2A**) morphologically identical to those observed associated with the bacteria (**Fig. 1A**). The approximate yield of Mtp from one hundred 7H11 agar plates was 14 mg of total protein, as determined by absorbance at OD_{280} as compared to a bovine serum albumin (BSA) standard curve. A page with our present procedure to purify *M. tuberculosis* pili is included at the end of this section.

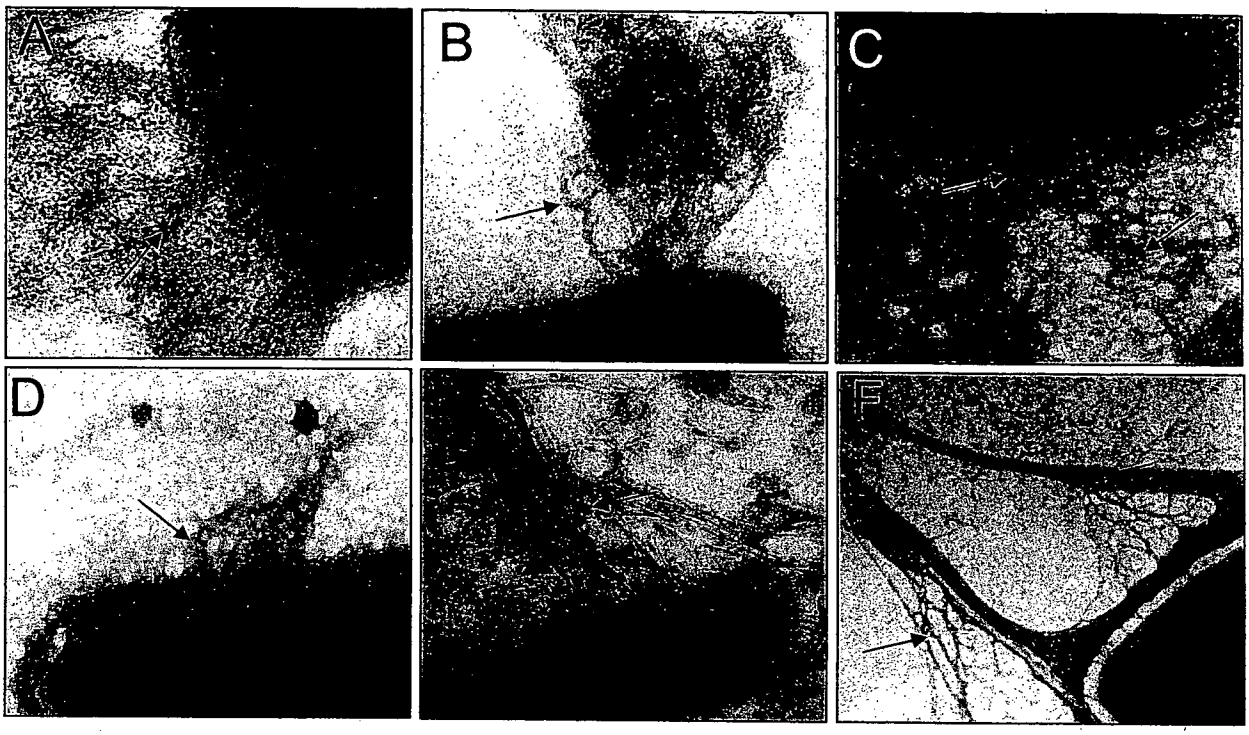

Figure 1. *Mycobacterium* species produce pili. Electron micrographs showing different pili morphotypes produced by *M. tuberculosis* H37Ra (**A**) (x28000); *M. tuberculosis* H37Rv (**B**) (x25000); *M. tuberculosis* CDC1551 (**C**) (x22000); *M. bovis* BCG (**D**) (x30000); *M. smegmatis* 1-2c (**E**) (x25000); and *M. fortuitum* (**F**) (x25000). Arrows point to the fibers produced by the various strains tested.

4. Identification and purification of *M. smegmatis* pili (Smp). In order to determine if the Mtp pili structures produced by *M. tuberculosis* were biochemically or antigenically related to *M. smegmatis* pili (Smp), we also purified pili from *M. smegmatis* employing a similar procedure as described above for Mtp. The final pili preparation obtained was rich in pili aggregates or bundles, as determined by TEM (**Fig. 2B**). The approximate yield of Smp from a one-liter culture was 3 mg of total protein. It is noteworthy to mention that the morphology of the purified Mtp and Smp is different. Further studies, presented later in the Preliminary Studies, demonstrate that they are also antigenically different.

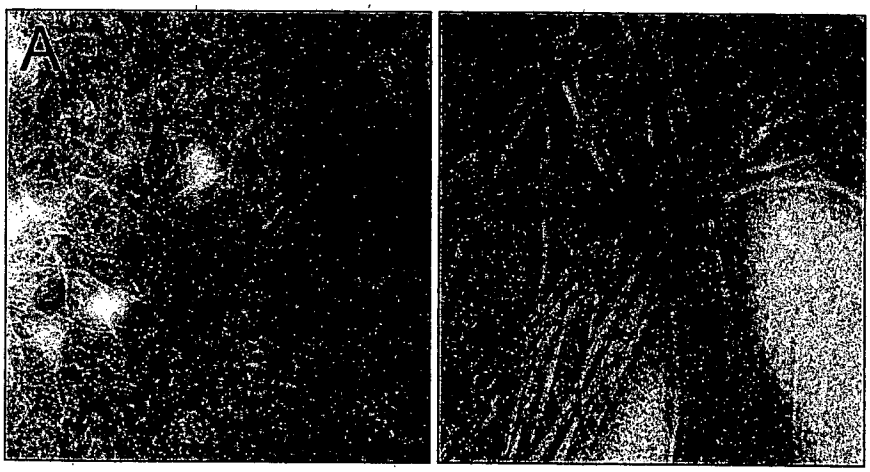

Figure 2. Electron micrographs of mycobacterial pili extracts. A, Purified pili from *M. tuberculosis* H37Ra (x25000); **B,** Purified pili from *M. smegmatis* 1-2c (x40000). Note the different morphology of the fibers.

5. **Initial biochemical characterization of mycobacterial pili.** Generally, pilin monomers are proteins in the range of 14-25 kDa that can be identified in SDS-PAGE gels after dissociation of the pili filaments under conventional denaturing (in the presence of SDS, 2-mercaptoethanol, and boiling at 100°C) conditions. However, some bacterial pili types require special chemical treatments such as acidification (pH 1.5) and boiling with HCl, as in the case of the common *E. coli* type I pili, or denaturation with formic acid, as in the case of the *Salmonella* and *E. coli* curli fibers. We began our characterization of purified Mtp and Smp preparations in 16% SDS-polyacrylamide gels under normal denaturation conditions. After repeated efforts employing Coomasie Blue and silver staining techniques, we were unable to detect the presence of polypeptide bands in the pili samples in the range of 14 to 25 kDa that correlated with the presence of abundant pili in samples as demonstrated by TEM. The absence of other high MW polypeptide bands and TEM analysis indicated that our pili preparations were relatively pure (data not shown). This suggested that both Mtp and Smp were highly hydrophobic and non-dissociable under regular 2-mercaptoethanol and SDS-PAGE denaturation. In fact, we observed the presence of protein aggregates in the wells of the stacking gel confirming that the pili filaments were still intact and therefore the pilins were not entering the gel. Nevertheless, the pili fractions were subjected to N-terminal amino acid sequencing by Edman degradation at the Protein and Nucleic Acid Facility at Stanford University. Both Mtp and Smp were blocked in their N-terminus. Further efforts employed several different chemical, physical and enzymatic treatments to allow dissociation of the pili aggregates followed by denaturation of the pilin proteins by SDS-PAGE.

Isolated Mtp and Smp were treated with various chemical reagents that have been used by other groups to resolve pili into their pilin monomers. Formic acid treatment was used in the manner described by Collinson *et al.*. Approximately 0.10 mg of the pili preparation was dried using speed-vacuum centrifugation, resuspended in 95% formic acid, and immediately frozen at –70°C. After 1 h, the sample was dried by heating for 96 h to remove all traces of acid. Pili were also incubated in the presence of 0.1% to10% SDS at 37°C for 18 h. Attempts were also made to dissolve the fibers in 2 to 8 M urea or saturated guanidine-HCl for 18 h at 37°C. The purified pili preparations were also incubated in 0.1, 1, and 10% Triton X-100 at 37°C for 18 h. The purified pili were subjected to acid (pH 1.8) or alkaline (pH 12.0) treatments and boiled for 30 min as previously described. The pili fibers were also treated with 0.5%, 1%, and 5% sodium deoxycholate and incubated at 37°C for 18 h. After the above treatments samples were either prepared for separation by SDS-PAGE or for viewing by TEM. After electrophoresis of all above treated Mtp or Smp pili samples, material was still observed in the wells of the stacking gel and no visible protein bands were detected in the separating gel following either Coomasie Blue or silver staining methods. Similar results were observed when a 10% resolving gel was also used (data not shown). After all of the above chemical treatments pili fibers were still visible by TEM. These results demonstrate that *M. tuberculosis* and *M. smegmatis* pili are very stable and cannot be broken down into their pilin subunits by methods conventionally utilized by other research groups working with pili.

This extreme stability of the purified mycobacterial pili is not unique and has been reported for the pili of the Gram positive bacterium *Streptococcus salivarius* and also for the pili of *Actinomyces* species. The *Actinomyces* fibers do not dissociate into subunits after SDS-PAGE and the biochemical characterization and identification of the fibril subunit and genes was only accomplished by expressing *A. naeslundii* genes in *E. coli* and detecting their expression using anti-fimbrial sera. The Gram negative pathogen *Salmonella enteritidis* produces curli which require extraordinary denaturation methods to dissociate and visualize the curlin subunit in SDS-PAGE gels.

Further, the purified pili from *M. tuberculosis* and *M. smegmatis* were treated with a variety of enzymes to determine their biochemical nature. Pepsin, trypsin, or proteinase K treatment under the appropriate enzymatic conditions and concentrations, as observed by SDS-

PAGE analysis and TEM, did not degrade the pili. Due to the aggregative nature of the pili, amino acid residues of the macromolecule may not be accessible for cleavage by these proteases. Treatment of Mtp with the enzyme cellulase had no effect, demonstrating that the pili are not composed of cellulose polymers . The pili were also incubated at 37°C for 18 h with lysozyme (10 mg/ml) without effect, indicating the fibers are not polymers of peptidoglycan. The purified pili were extracted with 2:1 chloroform:methanol and found to remain in the interface after centrifugation. This indicates that the pili are not a non-polar lipid substance from the mycobacterial cell wall.

6. **Detection of antibody to *M. tuberculosis* pili in sera from tuberculosis patients.** An initial study was done to determine if anti-pilus antibody is present in sera from human patients with active tuberculosis infections. If such antibody could be detected, it would suggest that *M. tuberculosis* pili are produced *in vivo* during natural human infections and that they are antigenic. Toward this aim, sera from thirty-six cavitary tuberculosis patients, admitted to the Instituto Mexicano del Seguro Social, Monterrey, Mexico and sera from five healthy controls were obtained from Dr. Guillermo Caballero Olin for these studies. The sera were tested against purified Mtp preparations obtained from H37Ra using immunofluorescence (IF) as previously described. Briefly, glass cover slips were prepared with a diluted pili preparation, air-dried, and fixed with PBS containing 3% formaldehyde overnight at 4°C. The coverslips were washed with PBS followed by incubation at room temperature for 1 h in sera diluted 1:1,000 in PBS containing 10% fetal calf serum (PBS/FCS). After thorough washing with PBS, to remove unbound antibody, the samples were incubated for 1 h with goat anti-human IgG Alexa Fluor 488 (Molecular Probes) diluted 1:5,000 in PBS/FCS. The coverslips were washed and mounted on glass slides before observation under a Nikon TE 2000S fluorescent microscope using Metacam software. Strikingly, we found that a high percentage (60%) of the sera from tuberculosis patients reacted very strongly with the purified pili preparation (**Fig. 3A, B, C, and D**). We considered the reaction positive when long fluorescent coiled fibers were observed after incubation with the patients' sera. No fluorescent filaments were observed with the goat anti-human IgG Alexa Fluor 488 alone, confirming the specificity of the reaction (data not shown). More interestingly, 5 of 5 sera from healthy human controls did not react with Mtp fibers (**Fig. 3E**).

Further evidence that TB patient sera contain antibodies that recognize Mtp fibers was obtained by ELISA assays. Microtiter plates were coated with purified Mtp fibers, incubated with serial dilutions of TB patient sera, followed by incubation with anti-human IgG HRP conjugate. The ELISA analyses indicated that 60% (n=36) had IgG titers greater than 3,200 while healthy control sera (n=5) did not react significantly with Mtp (**Fig. 4**). Nearly all the individual TB patient sera that reacted strongly with purified Mtp by IF, had anti-Mtp IgG titers higher than 3,200. **Taken together these data demonstrate that Mtp are produced by *M. tuberculosis* during natural human TB infections, indicating that they are expressed *in vivo*. The studies also show importantly that Mtp are antigenic, and the host immune response to Mtp may correlate with disease.**

7. **Are *M. tuberculosis* (Mtp) and *M. smegmatis* (Smp) pili antigenically related?** IF assays were done using purified Mtp and Smp to determine if they were antigenically similar. Four of the tuberculosis patient sera that reacted strongly with Mtp (**Fig. 3**) were incubated with Smp-coated glass cover slips, while rabbit anti-Smp serum was incubated with Mtp-coated glass cover slips following IF methods described above in section C6. It was observed that TB patient sera did not react with the Smp fibers nor did the rabbit anti-Smp serum react with Mtp fibers (data not shown). Therefore these preliminary results suggest that the Mtp and Smp pili are immunologically distinct.

8. **Adherence of Mtp to extracellular matrix proteins.** Extracellular matrix proteins (ECM) such as fibronectin, collagen, laminin, and vitronectin act as interlinking molecules in connective tissues and are ideal microbial adhesion targets for colonization of host tissues. Studies were done to determine if Mtp has affinity for ECM. For these experiments a sandwich based ELISA assay was employed using 1.5 μg of Mtp immobilized onto ELISA microtiter plate wells and blocked with PBS Superblock (Pierce) prior to the addition of increasing concentrations of fibronectin, laminin, and collagen IV (Sigma). After incubation and wash steps the bound ECM proteins were detected using either a 1:5000 dilution of rabbit anti-fibronectin, anti-laminin, or mouse monoclonal anti-collagen IV antibodies (Sigma). The anti-ECM antibodies were detected using a 1:5000 dilution of anti-rabbit or anti-mouse peroxidase conjugates (Sigma) and this complex was detected using a TMB single solution substrate (Zymed). The reaction was stopped with 1N HCl, and absorbance was read at 450 nm using a microtiter plate reader. The results indicate that purified Mtp fibers bind laminin and fibronectin in a dose-dependent manner, and do not significantly bind to collagen IV (**Fig. 5**). Clearly Mtp has a strong affinity for binding to laminin and to a lesser extent fibronectin.

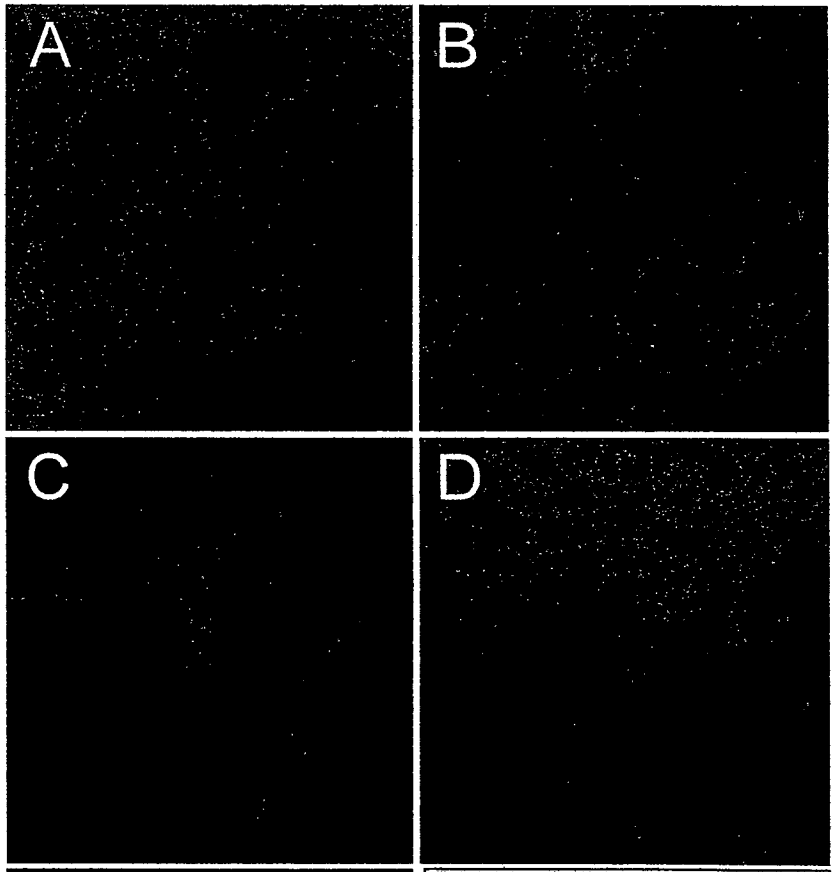

Figure 3. Reaction of TB patient sera with purified Mtp. Mtp (Fig. 2A) were reacted with human TB patient sera HS14, (**A**), HS7 (**B**), HS35 (**C**), HS29 (**D**), and healthy control sera (**E**) and detected with anti-human IgG FITC-conjugate. Positive reactions (**A-D**) are depicted by the presence of fluorescent fibers. No reactivity was observed with healthy control sera (**E**). Magnification of fluorescence micrographs A-E x1000.

The binding of the pili to laminin strongly implicates Mtp as an adhesin for *M. tuberculosis.* These results provides preliminary evidence for the role of Mtp in human disease as ECM proteins are important elements in the epithelial substrata of the lung and other organ systems which can be exposed during tissue damage due to an inflammatory response to a TB infection. Thus, Mtp may play a role in *M. tuberculosis* adherence and colonization in the lung during infection.

9. **Ultrastructural analysis of biofilm-like communities and demonstration of fibrillar structures.** Many environmental and pathogenic bacteria live in communities tightly associated to biotic and abiotic surfaces. These so-called biofilms are intrinsic ways in which the bacterial pathogens protect themselves from the bactericidal activity of antimicrobial drugs or from the clearance mechanisms of the body. There are virtually no reports on the capacity of *M.*

tuberculosis to form biofilms. Plate-grown *M. tuberculosis* H37Ra was washed in HBSS and seeded at 10^7 bacteria/ml onto glass cover slips in RPMI. We captured the bacilli attaching to the glass substratum after incubation for 3 h at 37°C in a 5% CO_2 atmosphere, followed by fixation with 3% formalin, and processing for scanning electron microscopy (**Fig. 6**). The bacilli appeared to produce a massive meshwork of fibrous matter (resembling pili) that tethers the bacteria, which we presume, allows the formation of biofilm-like communities. Pili, such as type IV and curli, have been implicated in biofilm formation. The nature and identity of these fibrous structures is of course unknown but we are aiming to characterize them further, in particular to determine whether these fibers are related to Mtp. Once specific anti-Mtp antibodies become available we will be able to test this possibility and determine if Mtp are involved in biofilm formation.

Summary. It is tempting to speculate that production of pili by *M. tuberculosis* would be of critical importance for the pathogen to interact with target sites on the surface of epithelial cells and macrophages to favor establishment of the pulmonary disease. Several important considerations arise from these preliminary data: **i)** that *M. tuberculosis* does produce pili which could contribute to the virulence properties of these bacteria; **ii)** that expression of pili is regulated by environmental and nutritional signals; **iii)** antibodies in TB patient sera recognize Mtp demonstrating they are produced during human infections; and **iv)** that pili might be involved in physical bridging between bacteria and host ECM in molecular cross-talk between mycobacteria and mammalian cells.

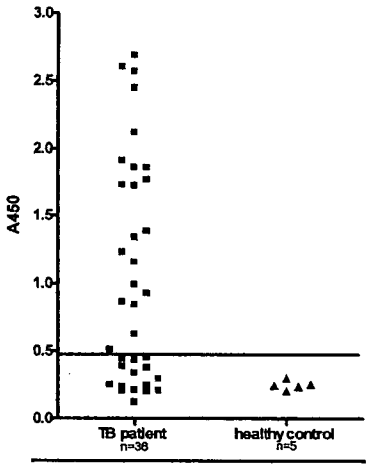

Fig. 4. Sera from TB patients react to purified Mtp. Sera from TB patients (n=36) and from healthy controls (n=5) were tested for the presence of anti-Mtp antibodies by ELISA using immobilized Mtp fibers. Most of the patient sera (60%) showed a significant titer against Mtp fibers. Results presented obtained at sera 1:3200 dilutions run in triplicate. The horizontal line indicates the cut-off value of two times the average ELISA titer A_{450} reading of healthy control sera.

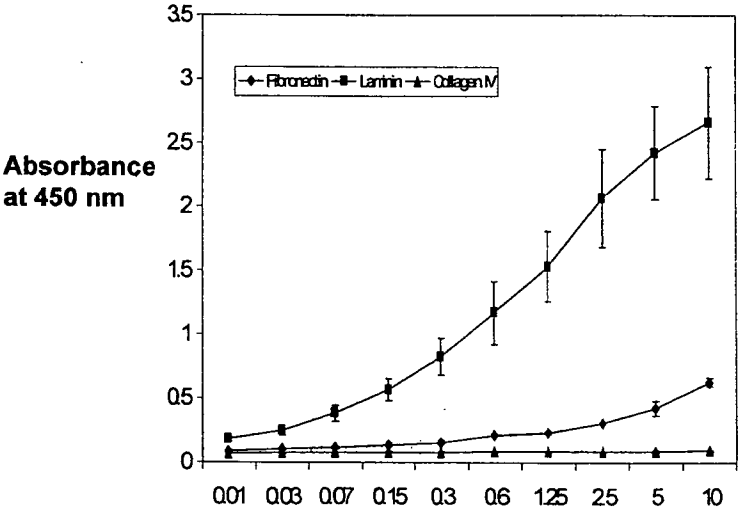

Figure 5. Mtp binds to extracellular matrix proteins. Shown are the results of binding of increasing concentrations of fibronectin, laminin, and collagen type IV to Mtp-coated ELISA plates. Binding was quantitated by ELISA at A_{450}. Results are presented from 3 independent experiments run in triplicate.

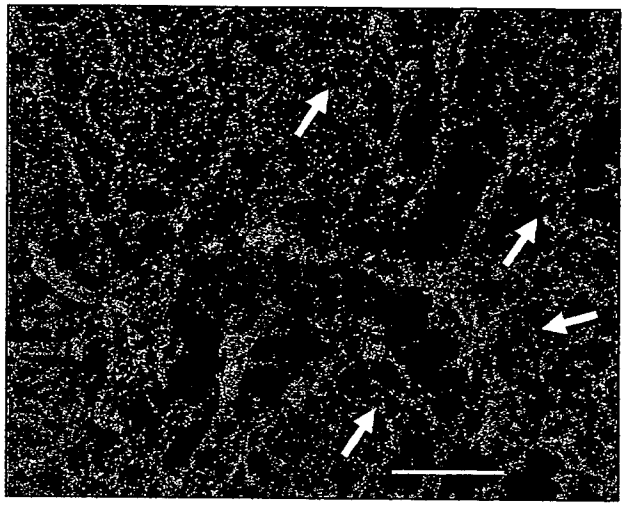

Figure 6. Scanning electron micrograph of *M. tuberculosis* H37Ra bacilli (red arrows) adhering to a glass substratum. Note the presence of a fibrillar meshwork (white arrows) tethering the bacteria, promoting bacterial interactions and the formation biofilm-like communities. Bar = 1 μm.

D. **What are the immediate and/or future applications?** The bacterium *Mycobacterium tuberculosis* is an important worldwide cause of pulmonary disease in humans. We have discovered a potential adherence factor called *M. tuberculosis* pili, or Mtp, which we hypothesize allows colonization and adherence of this microorganism to human eukaryotic cells in the lungs. Most importantly, 60% of the sera of humans with tuberculosis reacted with the purified pili, by both immunofluorescence and ELISA. This suggests that the pili are produced *in vivo* during natural infections and stimulate a humoral immune response. Because of these properties, this factor could be used in the development of a new vaccine to prevent tuberculosis in humans.

E. **Why is the subject of this disclosure better, more advantageous, than present technology? What are its novel or unusual features? What problems does it solve?** Bacterial structures called pili play a role in adherence of microorganisms to mammalian epithelial cells in a variety of diseases. Pili and other bacterial adherence factors have been successfully used in the development of effective vaccines against various infectious diseases. No pili have, until now, been identified in *M. tuberculosis*. Thus our discovery is new and novel because it is the first time that a pili-like adhesin has been identified in the human pathogen *M. tuberculosis*. A vaccine developed using purified Mtp could potentially induce antibody production that would block adherence of the bacilli to respiratory epithelial cells in humans and thus prevent initial colonization and infection. Thus, *M. tuberculosis* pili have great potential for use in the development of a new and possibly more effective vaccine against this highly deadly human disease. In the U.S.A. and all over the world there is a critical need for the development of more effective vaccines against tuberculosis. Major research groups in industry and at major academic institutions are actively pursuing research and development of new anti-tuberculosis vaccines. Additionally, *M. tuberculosis* pili may potentially be used in the development of new methods to diagnose cases of human tuberculosis.

What companies may be interested in this invention? Potential size of the market. Pharmaceutical and Vaccine Companies. As stated in the background section, almost one third of the world populations suffers of tuberculosis disease. Thus, the size of the market is quite large.

Purification of pili from *Mycobacterium tuberculosis* H37Ra*

This protocol may be modified in the future for optimal purification and recovery of pili fibers.

1. Dilute 1ml frozen stock of *M. tuberculosis* cells in 10 ml 7H9 + ADC containing 0.05% Tween-80 and incubate at 37° with shaking for 48-72 h to prepare starter culture.

2. Inoculate 100 Middlebrook 7H11 + glycerol agar plates with 100 μL starter culture of *M. tuberculosis* and incubate at 37° until a heavy lawn of growth is observed, 10-14 days. Remove loopful of growth to 1 ml dH_2O gently vortex to resuspend and prepare sample for TEM to verify production of pili fibers.

3. Harvest the plates, using a glass spreader, 15 plates into a beaker containing 60 ml 150 mM ethanolamine buffer pH 10.5. Portion the suspension into two 50 mL conical tubes and add 3mm glass beads to each tube. Repeat the procedure for the remaining plates, for 100 plates use approximately 250 ml ethanolamine buffer and 8 conical tubes. Vortex the tubes full power for 1 min, followed by vigorous shaking for 3-5 min and allow tube to settle for 1-2 min. Collect the upper 25-30 ml cell suspension to new tubes.

4. Centrifuge the suspension at 3000 rpm for 60 min to pellet the cells. Recover the supernatant fraction (3KS). Check both 3KS fraction and pellet fraction (3KP) by TEM to verify presence of pili in 3KS. If necessary resuspend 3KP in ethanolamine buffer, gently resuspend and centrifuge as described to recover pili that may have been sedimented with the cells. To limit loss of pili into the bacterial pellet, due to clumping of the cells, centrifugation speed was reduced and run time was increased. Repeat 3000 rpm spins for 30 min 2 more times.

5. (Optional) If large amounts of vesicular material is visible in 3KS by TEM, extract the final 3KS fraction with an equal volume of 2:1 chloroform: methanol by vigorously shaking for 5 min. Spin the suspension at 12000 rpm for 10 min to separate the phases. Recover the upper aqueous phase and the interphase that contains the pili fibers.

6. Load the pili fraction in thin-wall polyallomar tubes with anodized aluminum cap assemblies and concentrate fibers by ultracentrifugation at 120,000 x *g* for 3 hr at 4°C in a fixed angle rotor. Collect the supernatant as fraction 50KS and resuspend the pellet in sterile water as fraction 50KP. Observe both fractions by TEM. The 50KP fraction contains rich amounts of aggregated fibers while the supernatant fraction 50KS should be devoid of pili fibers. The approximate yield of protein (pili) from 100 plates by A280 is approximately 14 mg.

*Pili were first observed by TEM in *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra on 11-06-02, in *M. tuberculosis* clinical strain CDC 1551 on 12-09-02, and were first purified from *M. tuberculosis* H37Ra on 03-13-03.

UA04-020

Novel Pili of Mycobacteria

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Subject category D15 Cell surface structures of pathogenic microorganisms

A large number of microbial pathogens produce fibrillar surface structures termed pili. Pili often mediate the close interaction between the bacteria and host cells, thus they are important colonization and virulence factors. Here we report that the important human pathogen *Mycobacterium tuberculosis* produces pili. *M. bovis* BCG was also observed to produce pili. Additionally, it was found that both the fast-growing *M. smegmatis,* and the pathogenic *M. fortuitum* produce morphologically distinct pili from *M. tuberculosis*. Pili were isolated from both *M. smegmatis* and *M. tuberculosis* using mechanical shearing, differential centrifugation, and final purification over a CsCl density gradient. Immunofluorescent microscopy (IF) and ELISA were employed to evaluate the reactivity of antibodies from convalescent tuberculosis (TB) patient sera to purified *M. tuberculosis* pili (Mtp). It was found that 60% of TB patient sera (n=36) tested displayed intense epifluorescence when incubated with purified Mtp and had ELISA IgG titers against Mtp greater than 3,200. Healthy contol sera (n=5) did not react to the purified pili. The affinity of Mtp for the extracellular matrix proteins fibronectin, laminin, and type IV collagen was determined using an ELISA-based assay. We found that Mtp has a strong binding affinity for laminin and a weaker interaction with fibronectin suggesting a possible adherence mechanism for *M. tuberculosis* that would facilitate the interaction between the bacillus and host cells. Further, immunogold labelling experiments demonstrated that antibodies against purified pili from *M. smegmatis* do not react with Mtp suggesting that Mtp are antigenically distinct from *M. smegmatis* pili. The IF and ELISA data using TB patient sera are compelling evidence that the historically deadly human pathogen, *M. tuberculosis*, produces pili during human infection. In summary, this is the first report of pili being produced by mycobacteria and these results suggest that Mtp may be a newly identified adherence factor for the tubercle bacillus.

Commercial Potential of *M. tuberculosis* pili (Mtp) for Vaccine and Diagnostics Development

The bacterium *Mycobacterium tuberculosis* is an important worldwide cause of pulmonary disease in humans. We have discovered a potential adherence factor called *M. tuberculosis* pili, or Mtp, which we hypothesize allows colonization and adherence of this microorganism to human eukaryotic cells in the lungs. Most importantly, over 60% of the sera of humans with tuberculosis reacted with the purified pili, by both immunofluorescence and ELISA. This suggests that the pili are produced *in vivo* during natural infections and stimulate a humoral immune response. Also, additional studies have also demonstrated that Mtp also stimulates a strong proliferative T cell response (cell-mediated immune response, CMI) to this antigen using white blood cells derived from tuberculosis patients. Because of these properties, Mtp could be used in the development of a new vaccine to prevent tuberculosis in humans.

Bacterial structures called pili play a role in adherence of microorganisms to mammalian epithelial cells in a variety of diseases. Pili and other bacterial adherence factors have been successfully used in the development of effective vaccines against various infectious diseases. No pili have, until now, been identified in *M. tuberculosis*. Thus our discovery is new and novel because it is the first time that a pili-like adhesin has been identified in the human pathogen *M. tuberculosis*. A vaccine developed using purified Mtp could potentially induce antibody production that would block adherence of the bacilli to respiratory epithelial cells in humans and thus prevent initial colonization and infection. Thus, *M. tuberculosis* pili have great potential for use in the development of a new and possibly more effective vaccine against this highly deadly human disease. In the U.S.A. and all over the world there is a critical need for the development of more effective vaccines against tuberculosis. Major research groups in industry and at major academic institutions are actively pursuing research and development of new anti-tuberculosis vaccines.

Additionally, *M. tuberculosis* pili (Mtp) may potentially be used in the development of new methods to diagnose cases of human tuberculosis. Although many antigens of the bacillus have been evaluated, no specific test appears to have sufficient sensitivity or specificity to consistently detect cases of active tuberculosis. Thus there is a real need for the development of new assays that use either humoral or cellular immune response of the host to demonstrate the presence of active TB infection or disease. Since *M. tuberculosis* pili can stimulate both a humoral and CMI response during human disease, it suggests that the antigen may be a good candidate for use in the development of new immunologic diagnostic tests for the detection of active TB cases.

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Mycobacterium tuberculosis is the bacterial agent responsible for human pulmonary tuberculosis. For many bacterial pathogens, the ability to produce adhesins in the form of hair-like structures, called pili that mediate association with host cells. However, in the case *M. tuberculosis* no pili structures have ever been observed. We have, for the first time, identified and partially characterized pili-like structures on *M. tuberculosis* strains as fibers that associate into a highly hydrophobic meshwork of variable dimensions. These pili, herein called *M. tuberculosis* pili (or Mtp), are produced *in vitro* and were demonstrated to react with antibodies present in human convalescent sera obtained from tuberculosis patients. These novel findings are the basis for the present proposal, and may have important implications in terms of immunoprophylaxis, prevention and diagnosis of this historically deadly disease. The long-term objectives of this proposal are to advance knowledge of *M. tuberculosis* pathogenesis by elucidating the role of Mtp pili in the context of the interaction of the tuberculosis bacilli with host cells. The proposed specific aims are to:

1. **To Characterize the Biochemical Properties of Mtp.** The primary focus of this aim is the identification of the pilin monomer (MtpA). Purified fibers will be subjected to chemical and enzymatic treatments and Mtp peptide fragments isolated by HPLC. Fragments will be subjected to Edman degradation to identify the primary amino acid (AA) composition of MtpA. Liquid chromatography-mass spectrometry studies will assist in determining the mass of MtpA and its peptides. The AA information obtained will help to identify the open reading frame encoding the MtpA pilin gene, *mtpA*, in the *M. tuberculosis* genomic sequence. Liquid chromatography–tandem mass spectrometry will be useful in identifying peptide fragments and their possible post-translational modifications, thereby giving insight into the structure and function of the pili.
2. **Clone and Characterize the Gene(s) Encoding Mtp.** We will identify, clone, and characterize the genetic determinant involved in production of Mtp. Pili-deficient mutants will be constructed in the structural pilin gene as well as in key pili bioassembly genes and introduced into the chromosome of *M. tuberculosis* by allelic exchange. These mutants will then be used in studies proposed in Specific Aims No. 3 and No. 4 of this proposal.
3. **Define the Relationship Between Expression of Pili and the Ability of *M. tuberculosis* to Adhere, Enter and Survive Intracelluarly within Host Cells.** In this aim we are proposing to address specific questions regarding the biological function and role of Mtp in adherence and interaction of the bacteria with host epithelial cells and macrophages. The ability of pili knock-out to adhere, invade, and survive within cultured A549 respiratory epithelial cells, U-937 macrophages, and human mononuclear phagocytes will be evaluated as compared to the parental strain. We will also study other biological properties associated with other well-characterized pili systems such as bacterial aggregation and the ability of Mtp to stimulate production of pro-inflammatory molecules.
4. **Effect of Pili Gene Inactivation on Survival and Multiplication of *M. tuberuclosis* and the Evaluation of Protective Efficacy of Mtp in Mice.** The ability of the *M. tuberculosis* pili mutant to colonize, persist and replicate *in vivo* in a mouse respiratory aerosol infection model will be appraised as compared to the wild-type virulent parental strain. Studies will also be done to investigate the potential protective properties of Mtp against tuberculosis infections in mice.

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KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

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Sheldon L. Morris, Ph.D.	FDA	Collaborator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

This document contains confidential and privileged information that is currently under review regarding intellectual property protection by the University of Arizona. The following materials are respectfully submitted to be viewed only by members of this study section and others at the National Institutes of Health associated with this confidential process.

A. Specific Aims. *Mycobacterium tuberculosis* is the bacterial agent responsible for human pulmonary tuberculosis. Almost one third of the world's population suffers from this infectious disease. The *M. tuberculosis* bacillus is highly infectious and is spread by aerosols from infected individuals with active pulmonary disease. After inhalation of the bacteria, they are engulfed by alveolar macrophages, resist killing, and are able to survive within host macrophages. For many bacterial pathogens, the ability to produce proteinaceous adhesins in the form of hair-like structures called pili, that mediate their close interaction with the host cells, is an attribute of their pathogenic scheme. **We have, for the first time, identified and partially characterized pili-like structures on *M. tuberculosis* strains as 2-5-nm-wide flexible, coiled fibers that associate into a highly hydrophobic meshwork of variable dimensions. These structures, herein called *M. tuberculosis* pili (Mtp), are produced at 37°C *in vitro*, bind to the extracellular matrix proteins, and were demonstrated to react with antibodies present in human convalescent sera obtained from tuberculosis patients.** These novel findings are the basis for the present proposal, and may have important implications in terms of immunoprophylaxis, prevention, and diagnosis of this historically deadly disease. The long-term objectives of this proposal are to advance knowledge of *M. tuberculosis* pathogenesis by elucidating the role of Mtp pili in the interaction of the tuberculosis bacilli with human epithelial cells and macrophages. **The primary focus of this grant application is to further characterize the pili-like structures of *M. tuberculosis* at both the biochemical and genetic level and to determine their potential role in pathogenesis.** The proposed specific aims are:

1. **To Characterize the Biochemical Properties of Mtp.** In this aim the primary focus will be on the identification of the pilin monomer of Mtp (MtpA). Purified Mtp fibers will be subjected to chemical and enzymatic treatments and Mtp peptide fragments will be isolated by high pressure liquid chromatography (HPLC). These fragments will be subjected to Edman degradation to identify the primary amino acid composition of Mtp. Liquid chromatography-mass spectrometry studies will assist in determining the mass of MtpA and its peptides. The amino acid sequence information obtained by these methods will help to identify the open reading frame encoding the Mtp pilin gene, *mptA*, in the *M. tuberculosis* genomic sequence. Liquid chromatography-tandem mass spectrometry will be particularly useful in identifying peptide fragments and their possible post-translational modifications and thus give insight into the structure and function of the pili.
2. **To Clone the *mtpA* Gene and Construct Mtp-deficient Mutants.** Based on information obtained in Specific Aim No. 1 on the identity of *mtpA*, the gene will be cloned into the vector pOLYG. Mtp-deficient mutants in both virulent and avirulent *M. tuberculosis* will be constructed by allelic exchange recombination technology. Mtp-deficient mutants complemented with wild-type *mtpA* will also be constructed. The genomic regions surrounding *mtpA* will be analyzed for the presence of other genes potentially involved in pili biogenesis. The *mtpA* mutants and complemented mutants will then be used in studies proposed in Specific Aims No. 3 and No. 4 of this proposal.
3. **To Define the Relationship Between Expression of Pili and the Ability of *M. tuberculosis* to Adhere, Enter, and Survive Intracelluarly within Host Cells.** In this aim we are proposing to address specific questions regarding the biological function and role of Mtp in adherence and interaction of the bacteria with host epithelial cells and macrophages. Most importantly, 1) What is the function or biological relevance of Mtp production? 2) What is their contribution to the virulence of *M. tuberculosis*? The ability of pili-deficient mutants and complemented mutants (obtained in Specific Aim No. 2) to adhere, invade, and survive within cultured A549 respiratory epithelial cells, U-937 macrophages, and human mononuclear phagocytes will be evaluated as compared to the parental strain. By using Mtp-expressing bacteria, isogenic mutants unable to express Mtp, and complemented strains, we will be able to elucidate the contributive adhesive properties of Mtp in these processes. Biological properties associated with other pili will be studied in Mtp. These pili phenotypes include bacterial aggregation, hemagglutination, binding to extracellular matrix proteins, and the ability of Mtp to stimulate production of pro-inflammatory molecules.

4. To Investigate the Effect of Pili Gene Inactivation on Survival and Multiplication of *M. tuberculosis* in Mice and to Evaluate the Protective Efficacy of a Mtp vaccine. The ability of the *M. tuberculosis* pili mutant to colonize, persist and replicate *in vivo* in a mouse respiratory aerosol infection model will be appraised as compared to the complemented strain and the wild-type virulent parental strain. Studies will also be done to investigate the potential protective properties of Mtp against tuberculosis infection in mice.

HYPOTHESIS: We hypothesize that the pathogenic capabilities of *M. tuberculosis* rely in part on the expression of pili structures that enhance the microbes' ability to efficiently colonize the human host. Mtp may serve as an accessory adhesin required for initial adherence and/or subsequent lung colonization. Results from such studies will add to our understanding of tuberculosis pathogenesis and may lead to the development of improved vaccines and to more effective treatment, and prevention of this deadly disease.

B. BACKGROUND AND SIGNIFICANCE.

Tuberculosis Mortality and Morbidity. Tuberculosis is the predominant bacterial infectious disease that afflicts mankind. A recent World Health Organization report estimates that 1.86 billion people are infected with tuberculosis (32% of the world's population) and that in 1997, eight million new cases occurred (9). Over three million people die yearly from tuberculosis, the largest single infectious cause of mortality worldwide (1, 2, 10). Tuberculosis is still a persistent health problem in the U.S.A. due in part to the human immunodeficiency virus (HIV) epidemic. AIDS patients are highly susceptible to infection with *M. tuberculosis* and other non-tuberculosis mycobacteria that seldom infect individuals with intact immune systems (3). The increased incidence of tuberculosis has contributed to the emergence of multidrug resistant strains of *M. tuberculosis* (4). Such strains constitute a very serious problem because they cannot be easily treated with many of the most commonly used anti-tuberculosis antibiotics, resulting in high mortality and rates of transmission.

Molecular Pathogenesis of *M. tuberculosis*. *M. tuberculosis* is highly infectious and is spread by aerosols from infected individuals with active pulmonary disease. The bacilli are inhaled into the lungs and are engulfed by alveolar macrophages residing in the lower reaches of the respiratory tree. The microbes are highly resistant to killing by host macrophages and multiply within these phagocytes. The microorganism is internalized into macrophages via interactions of the phagocyte's complement C3 receptor and mannose receptor with *M. tuberculosis* (5, 11, 12). Once internalized, the bacillus inhibits phagosome-lysosome fusion (6, 14, 15) and phagosome acidification (7, 13, 14, 15), events that are critical to the microbicidal activities of macrophages. Various mycobacterial cell wall components may be involved in these processes (16). Classic studies by Goren *et. al* (20) suggest a role for mycobacterial sulfatides in inhibition of the phagosome-lysosome fusion process. The cell wall-associated glycolipid lipoarabinomannan (ManLAM) of *M. tuberculosis* has been shown to scavenge toxic oxygen products produced by macrophages. This may allow the pathogen to resist anti-microbial killing by the phagocyte (21). Recent studies by Fratti *et al.* reported that ManLAM of *M. tuberculosis* actually interferes with phagosomal acquisition of lysosomal contents and blocks syntaxin 6 transfer from the trans-Golgi network. ManLAM appears to specifically inhibit the pathway dependent on phosphatidylinositol 3-kinase activity and phosphatidylinostiol 3-phosphate-binding effectors (22). Thus, ManLAM is one of the bacillus factors responsible for the blockage of phagosomal development and maturation. After inhibition of phagosome-lysosome fusion, the bacilli multiply within the macrophage leading to phagocyte lysis and dissemination of *M. tuberculosis* to other host cells and sites throughout the body.

Although tuberculosis is now recognized as a major public health problem world-wide, there is a need for more information on the basic molecular mechanisms of *M. tuberculosis* pathogenesis, its mechanisms of drug resistance, and immunity to this pathogen (23, 24). The specific molecular mechanisms *M. tuberculosis* uses in these processes are unknown, but recent reports have, for the first time, identified potential virulence factors of *M. tuberculosis* by use of molecular biology techniques (31). The DNA sequencing and annotation of classic virulent strain *M. tuberculosis* H37Rv and the clinical isolate CDC1551 genomes have added much to our general knowledge of the genetics of this microbial pathogen (29, 30).

Bacterial Adherence. Adherence to host tissues is an essential and complex first stage in bacterial colonization for the establishment of bacterial infectious disease. In many cases, adherence is mediated by one or more adhesins that can act simultaneously or in distinct steps of an infectious process (97). Adherence is considered an important virulence trait, because it enables bacterial pathogens to deliver toxins efficiently to host tissues, to interact closely with the cell membrane favoring intracellular penetration, to overcome peristaltic clearance, and to establish microbial communities in biological niches. Adhesins, in the form of pili or outer membranes proteins, may mediate direct or indirect binding to host cells. For intracellular pathogens

such as *M. tuberculosis*, *Legionella pneumophila*, *Haemophilus influenzae, Listeria monocytogenes, Salmonella, Shigella*, and *Yersinia*, penetration and survival within eukaryotic cells are key traits that determine their pathogenic scheme (32, 41, 42, 46, 52, 98, 99).

A great deal of information is available in terms of the interaction and trafficking of *M. tuberculosis* within cells of the immune system and epithelial cells. It is reasonable to presume that the bacteria are able to express surface molecules devoted to the specific recognition of unique or common receptor components present on target tissues. Nevertheless, the mechanisms underlying the adherence properties of *M. tuberculosis* to the first line of epithelial cells before interacting with professional phagocytes are just beginning to be unraveled. Analysis of the genome sequence of *M. tuberculosis* has revealed various genes coding for putative adhesins and invasins, yet no fimbrial adhesins have been described in *M. tuberculosis*. It is still unknown if these organisms colonize the respiratory epithelium of their human hosts prior to macrophage interactions in the alveolus. *In vitro*, multiple studies report that *M. tuberculosis* strains bind and/or invade several cultured non-phagocytic cell lines including A549 type II alveolar epithelial cells, HeLa epithelial cells, and CHO fibroblast cells (94-96). *M. tuberculosis* also disrupts rat alveolar epithelium, attaches, and invades human respiratory mucosa in organ cultures (46, 47, 96).

Pili of Gram Negative Bacteria. Bacteria pathogenic for mammalian and plant hosts have evolved virulence-associated adherence appendages called pili that mediate directly or indirectly the interaction of bacteria with specific host target cells (100, 101). Pili are proteinaceous polymeric structures generally composed of a major repeating subunit or pilin and, in some cases, a minor tip-associated adhesin subunit. The hydrophobic nature of pili overcomes the repulsive forces between bacteria and eukaryotic cells. Due to their key role in bacterial pathogenesis pili are viewed as virulence factors and therefore as important targets for vaccine development. Examples of pili as key players in the virulence scheme of pathogenic bacteria include: the filamentous hemagglutinin of *Bordetella pertussis* (93), the colonization factors of human and animal enterotoxigenic *E. coli* strains (102), the type IV pili of invasive and non-invasive Gram negative organisms (68, 101, 103-105), the pili of *Haemophilus influenzae*, and the repertoire of pili present in *E. coli* strains associated with urinary tract infections (106).

Structurally, pili or fimbriae are straight or flexible filaments 5-7 nm wide and 2-3 μm long while fibrillae are thinner fibers, 2-3 nm wide. Many genes are required for pili bioassembly and these encode the pilin major subunit, prepilin peptidase, chaperone, minor subunit (tip adhesin), proteins involved in membrane translocation, nucleotide-binding protein, channel-forming protein, among others. The best models so far studied of pili biogenesis are the P pilus of uropathogenic *E. coli* (UPEC) strains and type I pili (100, 107, 108). Pili have generally been associated with several biological activities such as agglutination of human and animal erythrocytes, bacterial adherence, and colonization of mucosal surfaces. Pili are classified into groups or families based on several criteria. These include morphology (e.g. curli and the bundle-forming type IV pili), association with disease (e. g. the pyelonephritis-associated pilus or Pap), biochemical and functional similarities (e. g. the type IV pili family, the CFA/I pili family), and receptor specificity [e. g. Dr adhesins] (100, 108).

The type IV pili family produced by many Gram negative bacterial pathogens has also been associated with virulence and other biological properties. These include twitching motility, bacterial aggregation, biofilm formation and development, colonization of host tissues, and DNA transformation (101, 109, 110). The type IV pili family shares in common a hydrophobic amino terminus and a typical signal peptide. They are often observed under the electron microscope as long bundles of filaments that resemble rope-like structures or wicks. They are found in pathogenic bacteria such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae, Dichelobacter nodosus, Vibrio cholerae*, *Pasteurella multocida, Aeromonas hydrophila*, as well as enteropathogenic and enterotoxigenic *E. coli* (68, 101, 104). The extended similarities between type IV pili suggest common functional features evolved for bacterial adaptation, survival, and virulence. Recent studies on the intracellular pathogen *Legionella pneumophila* have demonstrated the role of type IV pili in intracellular infection of human macrophages and in virulence (151, 152).

Closer examination of gene families involved in type IV pili biogenesis reveals how widespread these loci are in prokaryotic microorganisms. The γ-proteobacterial type IV pili cluster, the *tad-flp* locus of *Actinobacillus actinomycetemcomitans*, is present in a broad range of Gram negative and Gram positive bacteria (88, 89, 130). The bundled pili comprised of Flp monomers in *A. actinomycetemcomitans* are necessary for autoaggregation and adherence (88). These pili are virulence factors that likely contribute to the

microbes' ability to colonize human tissues. Disruption of the *flp1* pilin gene resulted in loss of fiber formation and the *flp* mutant was unable to colonize or persist in a rat model (89, 131).

Interestingly, approximately one half of the *tad* genes, including *flp1,* are clustered in a group of 12 homologous ORFs in the genome of *M. tuberculosis* H37Rv (130, 132). The genes in *M. tuberculosis* that are similar to *tad* genes include a peptide ABC transporter subunit, a putative ATPase homologous to TadA, as well as a hypothetical transmembrane and integral membrane proteins (29). Further, a conserved Flp pilin amino acid sequence is present in *M. tuberculosis* that contains the characteristic Flp motif including an invariant glycine residue preceding the putative leader peptide cleavage site, and a hydrophobic region which contains an invariant glutamate-tyrosine pair of amino acids (130). The annotation of the *M. tuberculosis* H37Rv genome also suggests that two of the genes in this putative *tad-flp* cluster are possible pilus assembly proteins (29). **We are currently investigating whether Mtp is biochemically related to Flp. We plan to construct mutants of the *M. tuberculosis flp* gene to determine if it is required for formation of the newly identified Mtp fibers.**

The curli (2-5 nm wide, coiled, highly aggregative) adhesive fibers are produced by commensal and some pathogenic *E. coli* isolates, and *Salmonella enterica* var. Typhimurium (111, 114). They have been associated with persistence in poultry infections, internalization into epithelial cells, and colonization of abiotic surfaces. Curli mediate development of biofilms by binding to certain host proteins including extracellular matrix proteins, plasminogen, and major histocompatibility complex class I molecules. They also induce cytokine activation during human sepsis (111-114).

Pili of Gram Positive Bacteria. Only a few Gram positive organisms have been reported to possess pili and only some have been characterized at the molecular and biochemical level. The lack of data regarding Gram positive pili illustrates the difficulty in finding homologous pili genes and proteins in *M. tuberculosis* or other mycobacteria by genome database searching, as has been successful for identifying pili in a number of Gram negative human pathogens (56, 57, 70-73). The closest phylogenetic relative possessing pili to mycobacteria is the *Corynebacterium* genus. Pili were observed on numerous pathogenic species of *Corynebacterium* by TEM and these pili were found to agglutinate trypsinized sheep erythrocytes (74-76). Although *Corynebacterium diphtheriae* is accepted to possess pili (77, 78), no biochemical characterization of the pilin subunit nor the genes involved in pili biogenesis in *Corynebacterium* have been identified. The pili of *C. diphtheriae*, by virtue of hemagglutination activities, indicate their ability to adhere to eukaryotic cell membranes making its pili a potential virulence factor. The presence of pili and non-fimbrial adhesins in this microbe may explain how the pathogen successfully colonizes the human throat.

Another Gram positive genus that has been observed to have pili are the oral *Streptococcus* species. *S. salivarius*, has been observed to have pili but attempts at dissociating the pili into subunits were unsuccessful employing commonly used techniques (66). Viridans streptococci are also known to produce pili (79). *S. parasanguis* uses a fimbriae-associated adhesin, Fap1, to form biofilms, while another fimbrial adhesin, FimA, is required to colonize prosthetic and natural heart valves (80, 81). Further studies on the Fap1 protein revealed that the fimbrial adhesin is a glycoprotein and monoclonal antibodies against the glycan moiety blocked *in vitro* adherence of *S. parasanguis* (119).

A substantial body of work exists for pili found on *Actinomyces naeslundii*, one of the Gram positive organisms that cause actinomycosis, a chronic disease characterized by suppurative abscesses and granulomas. *Actinomyces* exhibits two distinct fimbrial types, type 1 mediates adherence to proline-rich proteins in tooth enamel (82), and type 2 has lectin activity and mediates adherence to erythrocytes, epithelial cells, and PMNs (83). The two pili types are immunologically and functionally distinct (86). Recent genetic analysis on the *A. naeslundii* genome has demonstrated the presence of six ORFs required for fimbrial production flanking the type 1 subunit gene, *fimP*. This indicates for the first time in Gram positive organisms the potential of a pili gene cluster (87).

Mycobacterial Adherence Mechanisms. The initial interaction of *M. tuberculosis* with either macrophages or respiratory epithelial cells within the lungs requires adherence to these human host cell types. Recent work has begun to identify potential *M. tuberculosis* adhesins. Studies with human macrophages have identified ManLAM as an important cell wall surface ligand for binding to the mannose receptor of phagocytes that leads to phagocytosis of the pathogen (12, 32, 33). The terminal mannosyl units at the end of ManLAM are critical for binding to the mannose receptor, since AraLAM from avirulent mycobacteria, capped with arabinose in place of mannose, does not induce significant uptake into macrophages (12, 32, 33).

It has been thought that host alveolar macrophages are the key cell type in the initial stages of tuberculosis pathogenesis in the human host. It is envisioned that alveolar macrophages engulf and transport the microbe across the alveolar wall for systemic spread to the bloodstream. Yet, interestingly, since the alveolus is mainly composed of type I and type II pneumocytes and only contains 50 to 100 macrophages, *M. tuberculosis* has a greater probability to interact with these respiratory epithelial cells (34). Recent studies by several groups have reported that the microbe can invade and survive within alveolar epithelial cells (35-38). Bermudez and associates used a polarized bilayer system containing both human type II alveolar epithelial (A549 cells) and endothelial cells to investigate the mechanisms of *M. tuberculosis* translocation across the alveolar wall (38). Their studies showed that the pathogen can directly invade and translocate across epithelial cells and can also translocate within monocytes across the alveolar wall. Thus, the interactions and attachment of *M. tuberculosis* with epithelial cells may play an important role in TB pathogenesis.

Studies from the laboratories of Camille Locht and Michael Brennan have identified an adhesin on *M. tuberculosis* called the heparin-binding hemagglutinin adhesin (HBHA). This is a surface-exposed protein that is involved in binding of the microbe to epithelial cells but not to phagocytes (39-42). HBHA is a 22-kDa protein containing a carboxy terminal domain that is lysine rich and studies have demonstrated that this region binds to heparin sulfate-containing proteoglycans that are present on epithelial cells and on human type II pneumocytes (41). A *M. tuberculosis* HBHA-deficient mutant was constructed and tested in an intranasal mouse infection model (42). It was observed that the mutant colonized the lungs to levels equal to the wild-type parent, but it was significantly impaired in its ability to disseminate from the lungs to other regions of the body (42). These results suggest that HBHA is important in extrapulmonary spread and interactions with host epithelial cells. Antibody to the HBHA carboxy-terminal domain blocks binding of the protein to heparan sulfate-containing receptors on host cells and also impedes extrapulmonary spread of TB in the mouse model (42). This suggest that the humoral immune response to HBHA and possibly to other *M. tuberculosis* adhesins could potentially play a protective role in blocking TB dissemination from the lungs. Such antigens could be considered for inclusion in the development of new tuberculosis vaccines. Both *M. tuberculosis* (39, 44) and *M. leprae* (45) also produce homologous laminin-binding factors. Further studies are necessary to determine whether this laminin-binding adhesin may be important in mycobacterial disease pathogenesis.

Using a human organ culture system, Middleton *et al.* studied the interaction of *M. tuberculosis* and other mycobacteria with human respiratory mucosa (46, 47). They found that *M. tuberculosis* bound to the extracellular matrix (ECM) in areas of mucosal damage and not to ciliated cells. Preincubation of the bacteria with either fibronectin, *M. avium* fibronection attachment protein (FAP), or with *M. bovis* antigen 85B reduced adherence to extracellular matrix in a dose-dependent manner (46). When both FAP and antigen 85B were added together there was an additive inhibition of *M. tuberculosis* adherence. The authors conclude that both FAP and antigen 85B protein of *M. tuberculosis* bind to fibronectin exposed on ECM and may be important adherence factors in initial lung colonization.

Analysis of the *M. tuberculosis* genome identified the presence of a protein subfamily of acidic, glycine-rich proteins, called PE_PGRS that contains 67 members (50). Various groups have demonstrated by immunofluorescence and immuno-gold electron microscopy that some PE_PGRS proteins are localized on the cell surface of *M. tuberculosis* and *M. bovis* (51, 53). Studies using sera from human TB patients and from mice infected with *M. tuberculosis* detected production of antibodies against the PGRS domain but not the PE domain (50, 54, 55). These results provide evidence that some PE_PGRS are expressed during mycobacterial infections *in vivo*. Brennan and associates constructed a specific transposon insertion mutant in PE_PGRS gene *Rv1818* in *M. bovis* BCG and found that the mutant had altered surface properties that included less aggregation in liquid culture and reduced ability to infect J774 macrophages (53). Espitia *et al.* also reported that another PE_PGRS protein, produced by the gene Rv1759c of *M. tuberculosis*, was a fibronectin-binding protein that could mediate mycobacterial attachment to host cells (54). As it turns out this is the same fibronection-binding protein first reported by Abou-Zeid *et al.* (48). These studies taken together suggest that some PE-PGRS proteins may act as mycobacterial adherence factors.

C. PRELIMINARY STUDIES. Preliminary data presented below show that *M. tuberculosis* is able to produce surface appendages resembling pili. The criteria for calling these structures pili are: 1) their morphology and dimensions are similar to those of the pili of other bacteria, 2) using standard methods to isolate pili from other microorganisms, we have been successful in purifying these pili structures from *M. tuberculosis* and 3) Mtp binds to extracellular matrix proteins (ECM). These

observations may open a new avenue to further understand the pathogenic mechanisms of this deadly human pathogen.

1. ***Mycobacterium tuberculosis* produces pili structures.** In the course of ultrastructural studies of pathogenic and attenuated strains of *M. tuberculosis* by negative staining and transmission electron microscopy (TEM), we noted the presence of fibrillar structures resembling pili (also called fimbriae) when the bacteria were propagated under suitable laboratory growth conditions. Namely, cultures of various *M. tuberculosis* strains (avirulent H37Ra, virulent H37Rv and CDC1551) were grown on 7H11 agar plates containing OADC for three weeks at 37°C. All work with virulent *M. tuberculosis* strains was done in the BSL-3 laboratory of Dr. Friedman. Plate grown bacteria were gently suspended in 4% formaldehyde and incubated overnight in a microfuge tube. The bacteria were negatively stained with 1% phosphotungstic acid (pH 7.4) on Formvar-coated copper grids and then observed in a Phillips CM12 electron microscope at 80 kV. All of the *M. tuberculosis* strains analyzed produced thin (2-5 nm-wide), aggregative, flexible hair-like appendages that protruded several microns away from the bacterial cell surface (**Fig. 1A, B and C**). The fine fibrillar structures, **herein called *M. tuberculosis* pili or Mtp**, tended to aggregate to each other forming a meshwork of variable dimensions that appeared associated with the bacteria or free in the supernatants. As a particular note, these fibrillar structures are morphologically reminiscent of the well-characterized curli structures produced by some enteric bacterial pathogens (114). Under these growth conditions, ~5% of the bacterial cells present in the culture samples analyzed by TEM possessed pili. Studies were done to confirm that the pili structures observed were bacterial in nature and not artifacts present in the bacterial growth media employed. We prepared electron microscopy grids with liquid medium or with water sitting on solid agar medium that had been incubated for the same time as inoculated cultures. **In these control studies we did not observe any fibrillar structures, indicating that the fibers seen in *M. tuberculosis* cultures were of bacterial origin.**

We were then interested in studying the effect of culture conditions on the production of pili by H37Ra, H37Rv, and CDC1551. To this aim, we used a panel of liquid and solid media (**Table 1**) for bacterial growth and qualitatively determined the level of pili production by negative staining and TEM. *M. tuberculosis* strains were plated as a lawn and grown for a period of 3 weeks at 37°C in a 5% CO_2 atmosphere. *M. tuberculosis* broth cultures in 7H9 were grown for 2 to 3 weeks and GAS broth cultures were grown for 5 weeks until both reached an OD_{650} of 1.50. The data summarized in Table 1 indicates that Mtp are likely controlled by environmental stimuli since the level of pili production varied depending on the growth media used. Further, the qualitative analysis demonstrates that the avirulent strain of *M. tuberculosis*, H37Ra, has a diminished capacity to produce pili as compared to H37Rv and CDC1551. Strikingly, the greatest difference between virulent and attenuated *M. tuberculosis* was observed in broth grown cultures, where *M. tuberculosis* H37Ra produces nearly undetectable levels of pili, while *M. tuberculosis* H37Rv and CDC1551, the clinical isolate, produce the highest level of pili in the culture conditions tested. Ten to 25% of *M. tuberculosis* H37Rv and CDC1551 grown in either 7H9 or GAS broth contained pili as observed by TEM.

Table 1. *M. tuberculosis* pili production on various media as observed by TEM[1]

Strain	7H11 agar + OADC	3% Sheep Blood Agar	7H10 agar + glycerol	7H11 agar + glycerol	7H10 agar + glucose	RPMMA Agar***	7H9 broth + OADC + Tw*	GAS** broth + Tw*
H37Ra	+	++	+	++	+	-	-	-
H37Rv	+	++	+	+	+	-	+++	+++
CDC1551	+	++	+[2]	+[2]	+[2]	+	+++	++

[1] *All plate grown cultures in the table were incubated in a 5% CO_2 atmosphere. [2] Very limited growth was observed in these conditions. * Tween 80 (Tw); ** glycerol-alanine-salts (GAS) medium (58); *** RPMMA (reduced phosphate modified minimal A) agar is a defined minimal media on which mycobacteria can be grown (personal communication, James Megehee. **** 10 to 25% of M. tuberculosis contain pili. ** 5 to 10% bacteria contain pili. * less than 5% bacteria contain pili. - undetectable levels of pili.*

In conclusion, we have for the first time shown that *M. tuberculosis* produces pili structures and their production is under the control of environmental growth conditions.

2. **Production of pili by other *Mycobacterium* species.** We extended the TEM analysis to other *Mycobacterium* species, including *M. bovis* BCG, *M. fortuitum* (a clinical isolate), and *M. smegmatis* strain 1-2c to determine the production of pili after growth under different culture conditions as done with *M. tuberculosis*.

M. bovis BCG was grown as a lawn on 7H11 agar plates containing OADC for three weeks at 37°C, while *M. fortuitum* was grown in 7H9 broth containing glucose and Tween-80. *M. smegmatis* was grown in 7H9 broth cultures with and without Tween-80, LB broth, on 7H10 agar plates with either glucose or glycerol, sheep blood agar, RPMMA agar, or on LB agar plates.

We noted that all of these species were able to produce thin, flexible, pili when grown at 37°C (**Fig. 1D, E and F**). Interestingly, *M. smegmatis* produced long, semi-flexible pili structures that tended to aggregate laterally and form bundles or rope-like structures (**Fig 1E**). This is in contrast to *M. tuberculosis* strains that produced curli-like pili (**Fig 1A-C**). *M. smegmatis* produced pili regardless of the growth temperature (either at 25° or 37°C) and the composition of the media employed. However, the level of pili produced by *M smegmatis* was significantly higher in 7H9 broth supplemented with glucose, with or without Tween-80, and in Luria-Bertani broth. The *M. smegmatis* pili were also produced on solid media, albeit to a lesser extent than in liquid media. Growth in a 5% CO_2 atmosphere on agar plates did not stimulate increased production of pili by *M. smegmatis*. It is apparent that environmental cues are regulating the production of pili in *M. smegmatis* as was observed in *M. tuberculosis*. In conclusion, production of pili appears to be a generalized phenomenon in *Mycobacterium* species.

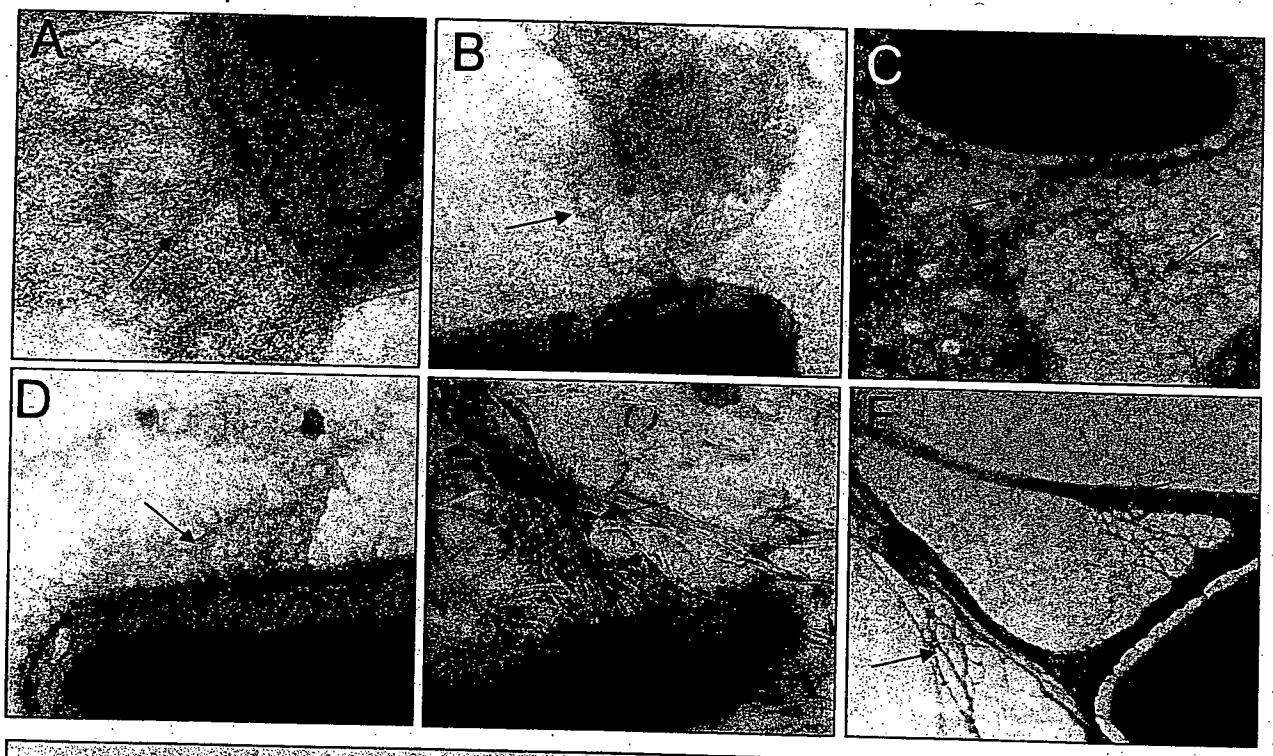


Figure 1. *Mycobacterium* species produce pili. Electron micrographs showing different pili morphotypes produced by *M. tuberculosis* H37Ra (**A**) (x28000), *M. tuberculosis* H37Rv (**B**) (x25000), *M. tuberculosis* CDC1551 (**C**) (x22000), *M. bovis* BCG (**D**) (x30000), *M. smegmatis* 1-2c (**E**) (x25000), and *M. fortuitum* (**F**) (x25000). Arrows point to the fibers produced by the various strains tested.

3. **Identification and purification of *M. tuberculosis* pili (Mtp).** Our next goal was to purify and identify the nature of the pili structures observed in *M. tuberculosis* cultures. For safety reasons and ease of working under non-BSL-3 conditions, *M. tuberculosis* H37Ra was used for pili purification. H37Ra was grown at 37°C under a 5% CO_2 atmosphere for three weeks on one hundred 7H11 agar plates supplemented with OADC (**Table 1**). Cultures were Gram stained and acid-fast stained to confirm purity of the bacterial preparations. The heavy bacterial lawn obtained was harvested from the plates into 150 mM mono-ethanolamine buffer (pH 10.5) and the pili were mechanically sheared from the surface of the bacteria. The bacteria were separated by repeated low speed centrifugation and the supernatant containing pili was extracted with 2:1 choloroform:methanol to remove vesicular material. The upper aqueous phase and interphase that contained pili fibers was recovered. This fraction was centrifuged at 18,000 x *g* to completely remove bacteria, bacterial membranes and debris. The supernatant fraction containing the pili was then recovered and concentrated by ultracentrifugation for several hours at 4°C. The pelleted pili was resuspended in PBS and dialyzed to remove any salts. This

preparation was further analyzed by TEM, which revealed the presence of abundant pili aggregates (**Fig. 2A**) morphologically identical to those observed associated with the bacteria (**Fig. 1A**). The approximate yield of Mtp from one hundred 7H11 agar plates was 14 mg of total protein, as determined by absorbance at OD_{280} as compared to a bovine serum albumin (BSA) standard curve.

4. Identification and purification of *M. smegmatis* pili (Smp). In order to determine if the Mtp pili structures produced by *M. tuberculosis* were biochemically or antigenically related to *M. smegmatis* pili (Smp), we also purified pili from *M. smegmatis* employing a similar procedure as described above for Mtp. The final pili preparation obtained was rich in pili aggregates or bundles, as determined by TEM (**Fig. 2B**). The approximate yield of Smp from a one-liter culture was 3 mg of total protein. It is noteworthy to mention that the morphology of the purified Mtp and Smp is different. Further studies, presented later in the Preliminary Studies, demonstrate that they are also antigenically different.

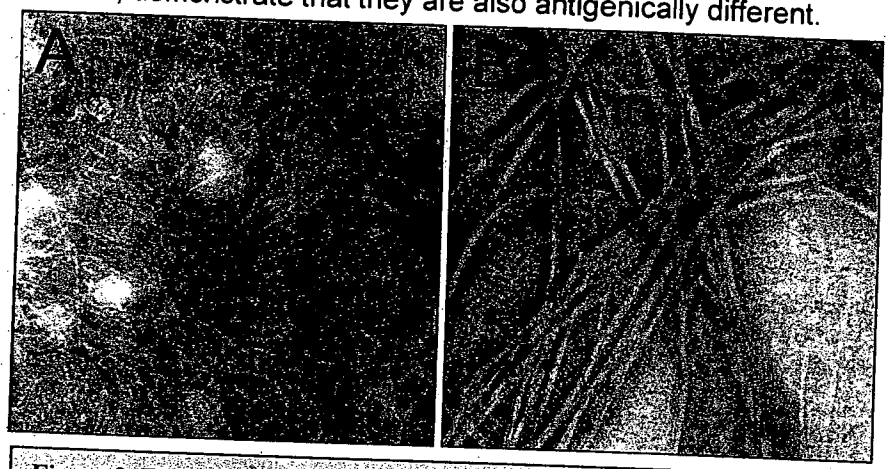


Figure 2. Electron micrographs of mycobacterial pili extracts. A, Purified pili from *M. tuberculosis* H37Ra (x25000); **B,** Purified pili from *M. smegmatis* 1-2c (x40000). Note the different morphology of the fibers.

5. Initial biochemical characterization of mycobacterial pili. Generally, pilin monomers are proteins in the range of 14-25 kDa that can be identified in SDS-PAGE gels after dissociation of the pili filaments under conventional denaturing (in the presence of SDS, 2-mercaptoethanol, and boiling at 100°C) conditions (115). However, some bacterial pili types require special chemical treatments such as acidification (pH 1.5) and boiling with HCl, as in the case of the common *E. coli* type I pili (116), or denaturation with formic acid, as in the case of the *Salmonella* and *E. coli* curli fibers (61, 111). We began our characterization of purified Mtp and Smp preparations in 16% SDS-polyacrylamide gels under normal denaturation conditions. After repeated efforts employing Coomasie Blue and silver staining techniques, we were unable to detect the presence of polypeptide bands in the pili samples in the range of 14 to 25 kDa that correlated with the presence of abundant pili in samples as demonstrated by TEM. The absence of other high MW polypeptide bands and TEM analysis indicated that our pili preparations were relatively pure (data not shown). This suggested that both Mtp and Smp were highly hydrophobic and non-dissociable under regular 2-mercaptoethanol and SDS-PAGE denaturation. In fact, we observed the presence of protein aggregates in the wells of the stacking gel confirming that the pili filaments were still intact and therefore the pilins were not entering the gel. Nevertheless, the pili fractions were subjected to N-terminal amino acid sequencing by Edman degradation at the Protein and Nucleic Acid Facility at Stanford University. Both Mtp and Smp were blocked in their N-terminus. Further efforts employed several different chemical, physical and enzymatic treatments to allow dissociation of the pili aggregates followed by denaturation of the pilin proteins by SDS-PAGE.

Isolated Mtp and Smp were treated with various chemical reagents that have been used by other groups to resolve pili into their pilin monomers. Formic acid treatment was used in the manner described by Collinson *et al.* (61). Approximately 0.10 mg of the pili preparation was dried using speed-vacuum centrifugation, resuspended in 95% formic acid, and immediately frozen at –70°C. After 1 h, the sample was dried by heating for 96 h to remove all traces of acid. Pili were also incubated in the presence of 0.1% to10% SDS at 37°C for 18 h (62). Attempts were also made to dissolve the fibers in 2 to 8 M urea or saturated guanidine-HCl for 18 h at 37°C (63). The purified pili preparations were also incubated in 0.1, 1, and 10% Triton X-100 at 37°C for 18 h. The purified pili were subjected to acid (pH 1.8) or alkaline (pH 12.0) treatments and boiled for 30 min as previously described (64, 116). The pili fibers were also treated with 0.5%, 1%, and 5% sodium deoxycholate and incubated at 37°C for 18 h (65). After the above treatments samples were either prepared for separation by SDS-PAGE or for viewing by TEM. After electrophoresis of all above treated Mtp or Smp pili samples, material was still observed in the wells of the stacking gel and no visible protein bands were detected in the separating gel following either Coomasie Blue or silver staining methods. Similar results were

observed when a 10% resolving gel was also used (data not shown). After all of the above chemical treatments pili fibers were still visible by TEM. These results demonstrate that *M. tuberculosis* and *M. smegmatis* pili are very stable and cannot be broken down into their pilin subunits by methods conventionally utilized by other research groups working with pili.

This extreme stability of the purified mycobacterial pili is not unique and has been reported for the pili of the Gram positive bacterium *Streptococcus salivarius* (66) and also for the pili of *Actinomyces* species (88). The *Actinomyces* fibers do not dissociate into subunits after SDS-PAGE and the biochemical characterization and identification of the fibril subunit and genes was only accomplished by expressing *A. naeslundii* genes in *E. coli* and detecting their expression using anti-fimbrial sera (84, 85). The Gram negative pathogen *Salmonella enteritidis* produces curli which require extraordinary denaturation methods to dissociate and visualize the curlin subunit in SDS-PAGE gels (61).

Further, the purified pili from *M. tuberculosis* and *M. smegmatis* were treated with a variety of enzymes to determine their biochemical nature. Pepsin, trypsin, or proteinase K treatment under the appropriate enzymatic conditions and concentrations, as observed by SDS-PAGE analysis and TEM, did not degrade the pili. Due to the aggregative nature of the pili, amino acid residues of the macromolecule may not be accessible for cleavage by these proteases. Treatment of Mtp with the enzyme cellulase had no effect, demonstrating that the pili are not composed of cellulose polymers (174). The pili were also incubated at 37°C for 18 h with lysozyme (10 mg/ml) without effect, indicating the fibers are not polymers of peptidoglycan. The purified pili were extracted with 2:1 chloroform:methanol and found to remain in the interface after centrifugation. This indicates that the pili are not a non-polar lipid substance from the mycobacterial cell wall.

6. Detection of antibody to *M. tuberculosis* pili in sera from tuberculosis patients. An initial study was done to determine if anti-pilus antibody is present in sera from human patients with active tuberculosis infections. If such antibody could be detected, it would suggest that *M. tuberculosis* pili are produced *in vivo* during natural human infections and that they are antigenic. Toward this aim, sera from thirty-six cavitary tuberculosis patients, admitted to the Instituto Mexicano del Seguro Social, Monterrey, Mexico and sera from five healthy controls were obtained from Dr. Guillermo Caballero Olin for these studies. The sera were tested against purified Mtp preparations obtained from H37Ra using immunofluorescence (IF) as previously described. (69). Briefly, glass cover slips were prepared with a diluted pili preparation, air-dried, and fixed with PBS containing 3% formaldehyde overnight at 4°C. The coverslips were washed with PBS followed by incubation at room temperature for 1 h in sera diluted 1:1,000 in PBS containing 10% fetal calf serum (PBS/FCS). After thorough washing with PBS, to remove unbound antibody, the samples were incubated for 1 h with goat anti-human IgG Alexa Fluor 488 (Molecular Probes) diluted 1:5,000 in PBS/FCS. The coverslips were washed and mounted on glass slides before observation under a Nikon TE 2000S fluorescent microscope using Metacam software. Strikingly, we found that a high percentage (60%) of the sera from tuberculosis patients reacted very strongly with the purified pili preparation (**Fig. 3A, B, C, and D**). We considered the reaction positive when long fluorescent coiled fibers were observed after incubation with the patients' sera. No fluorescent filaments were observed with the goat anti-human IgG Alexa Fluor 488 alone, confirming the specificity of the reaction (data not shown). More interestingly, 5 of 5 sera from healthy human controls did not react with Mtp fibers (**Fig. 3E**).

Further evidence that TB patient sera contain antibodies that recognize Mtp fibers was obtained by ELISA assays. Microtiter plates were coated with purified Mtp fibers, incubated with serial dilutions of TB patient sera, followed by incubation with anti-human IgG HRP conjugate. The ELISA analyses indicated that 60% (n=36) had IgG titers greater than 3,200 while healthy control sera (n=5) did not react significantly with Mtp (**Fig. 4**). Nearly all the individual TB patient sera that reacted strongly with purified Mtp by IF, had anti-Mtp IgG titers higher than 3,200. **Taken together these data demonstrate that Mtp are produced by *M. tuberculosis* during natural human TB infections, indicating that they are expressed *in vivo*. The studies also show importantly that Mtp are antigenic, the and host immune response to Mtp may correlate with disease.**

7. Are *M. tuberculosis* (Mtp) and *M. smegmatis* (Smp) pili antigenically related? IF assays were done using purified Mtp and Smp to determine if they were antigenically similar. Four of the tuberculosis patient sera that reacted strongly with Mtp (**Fig. 3**) were incubated with Smp-coated glass cover slips, while rabbit anti-Smp serum was incubated with Mtp-coated glass cover slips following IF methods described above in section C6. It was observed that TB patient sera did not react with the Smp fibers nor did the rabbit anti-Smp serum

react with Mtp fibers (data not shown). Therefore these preliminary results suggest that the Mtp and Smp pili are immunologically distinct.

8. Adherence of Mtp to extracellular matrix proteins. Extracellular matrix proteins (ECM) such as fibronectin, collagen, laminin, and vitronectin act as interlinking molecules in connective tissues and are ideal microbial adhesion targets for colonization of host tissues (168-171). Studies were done to determine if Mtp has affinity for ECM. For these experiments a sandwich based ELISA assay was employed using 1.5 μg of Mtp immobilized onto ELISA microtiter plate wells and blocked with PBS Superblock (Pierce) prior to the addition of increasing concentrations of fibronectin, laminin, and collagen IV (Sigma). After incubation and wash steps the bound ECM proteins were detected using either a 1:5000 dilution of rabbit anti-fibronectin, anti-laminin, or mouse monoclonal anti-collagen IV antibodies (Sigma). The anti-ECM antibodies were detected using a 1:5000 dilution of anti-rabbit or anti-mouse peroxidase conjugates (Sigma) and this complex was detected using a TMB single solution substrate (Zymed). The reaction was stopped with 1N HCl, and absorbance was read at 450 nm using a microtiter plate reader. The results indicate that purified Mtp fibers bind laminin and fibronectin in a dose-dependent manner, and do not significantly bind to collagen IV (**Fig. 5**). Clearly Mtp has a strong affinity for binding to laminin and to a lesser extent fibronectin.

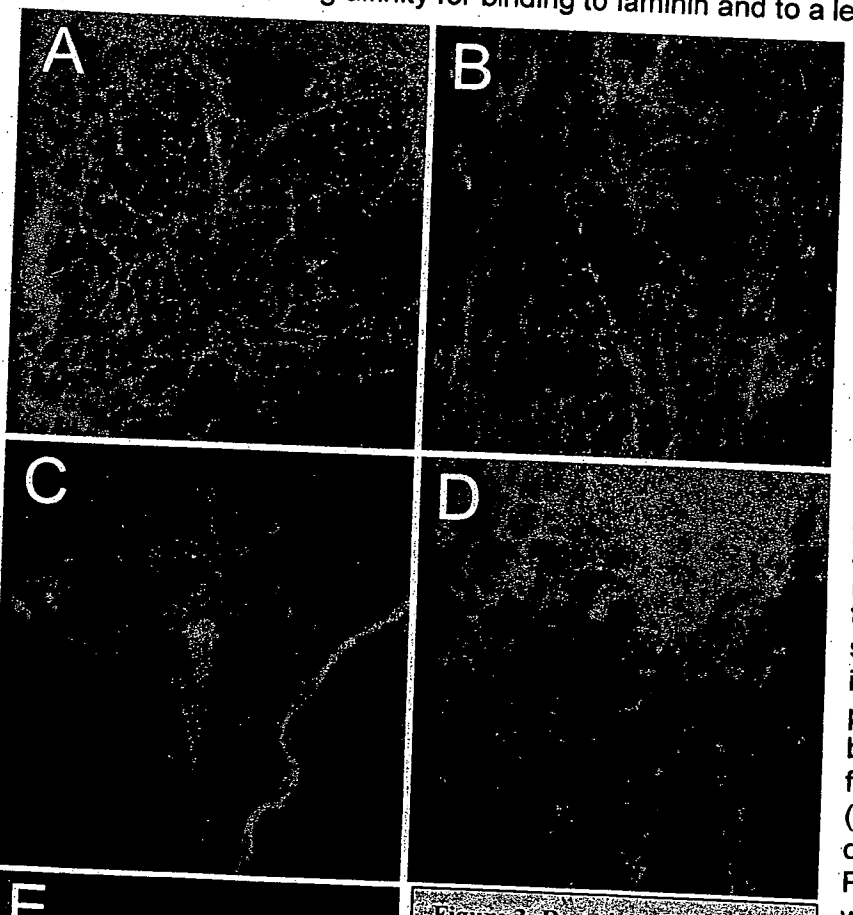


Figure 3: Reaction of TB patient sera with purified Mtp. Mtp (Fig. 2A) were reacted with human TB patient sera HS14 (**A**), HS7 (**B**), HS35 (**C**), HS29 (**D**), and healthy control sera (**E**) and detected with anti-human IgG FITC conjugate. Positive reactions (**A-D**) are depicted by the presence of fluorescent fibers. No reactivity was observed with healthy control sera (**E**). Magnification of fluorescence micrographs A-E x1000.

The binding of the pili to laminin strongly implicates Mtp as an adhesin for *M. tuberculosis*. These results provides preliminary evidence for the role of Mtp in human disease as ECM proteins are important elements in the epithelial substrata of the lung and other organ systems which can be exposed during tissue damage due to an inflammatory response to a TB infection (46, 47). Thus, Mtp may play a role in *M. tuberculosis* adherence and colonization in the lung during infection.

9. Ultrastructural analysis of biofilm-like communities and demonstration of fibrillar structures. Many environmental and pathogenic bacteria live in communities tightly associated to biotic and abiotic surfaces. These so-called biofilms are intrinsic ways in which the bacterial pathogens protect themselves from the bactericidal activity of antimicrobial drugs or from the clearance mechanisms of the body (49). There are virtually no reports on the capacity of *M. tuberculosis* to form biofilms. Plate-grown *M. tuberculosis* H37Ra was washed in HBSS and seeded at 10^7 bacteria/ml onto glass cover slips in RPMI. We captured the bacilli attaching to the glass substratum after incubation for 3 h at 37°C in a 5% CO_2 atmosphere, followed by fixation with 3% formalin, and processing for scanning electron microscopy (**Fig. 6**). The bacilli appeared to produce a massive meshwork of fibrous matter (resembling pili) that tethers the bacteria, which we presume, allows the formation of biofilm-like communities. Pili, such as type IV and curli, have been implicated in biofilm formation

(158-160). The nature and identity of these fibrous structures is of course unknown but we are aiming to characterize them further, in particular to determine whether these fibers are related to Mtp. Once specific anti-Mtp antibodies become available we will be able to test this possibility and determine if Mtp are involved in biofilm formation.

Summary. It is tempting to speculate that production of pili by *M. tuberculosis* would be of critical importance for the pathogen to interact with target sites on the surface of epithelial cells and macrophages to favor establishment of the pulmonary disease. Several important considerations arise from these preliminary data: **i)** that *M. tuberculosis* does produce pili which could contribute to the virulence properties of these bacteria; **ii)** that expression of pili is regulated by environmental and nutritional signals; **iii)** antibodies in TB patient sera recognize Mtp demonstrating they are produced during human infections; and **iv)** that pili might be involved in physical bridging between bacteria and host ECM in molecular cross-talk between mycobacteria and mammalian cells.

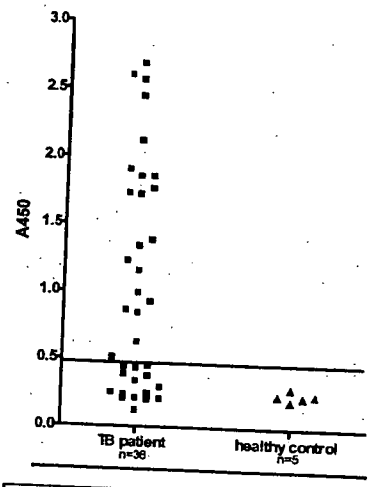


Fig. 4. Sera from TB patients react to purified Mtp. Sera from TB patients (n=36) and from healthy controls (n=5) were tested for the presence of anti-Mtp antibodies by ELISA using immobilized Mtp fibers. Most of the patient sera (60%) showed a significant titer against Mtp fibers. Results presented obtained at sera 1:3200 dilutions run in triplicate. The horizontal line indicates the cut-off value of two times the average ELISA titer A_{450} reading of healthy control sera.

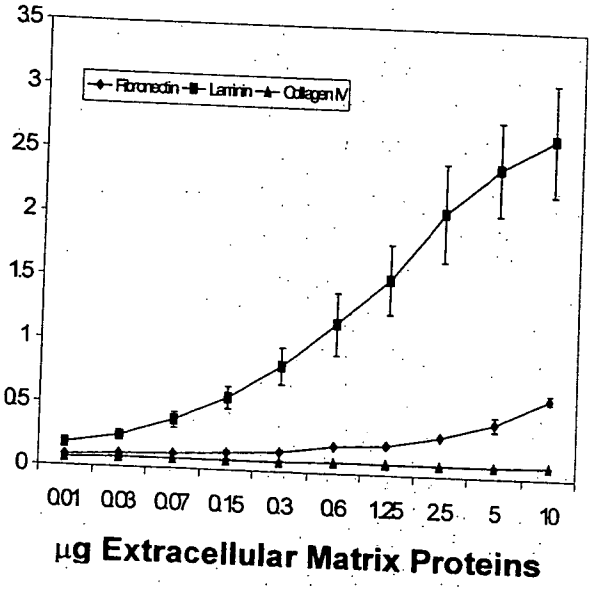


Figure 5. Mtp binds to extracellular matrix proteins. Shown are the results of binding of increasing concentrations of fibronectin, laminin, and collagen type IV to Mtp-coated ELISA plates. Binding was quantitated by ELISA at A_{450}. Results are presented from 3 independent experiments run in triplicate.

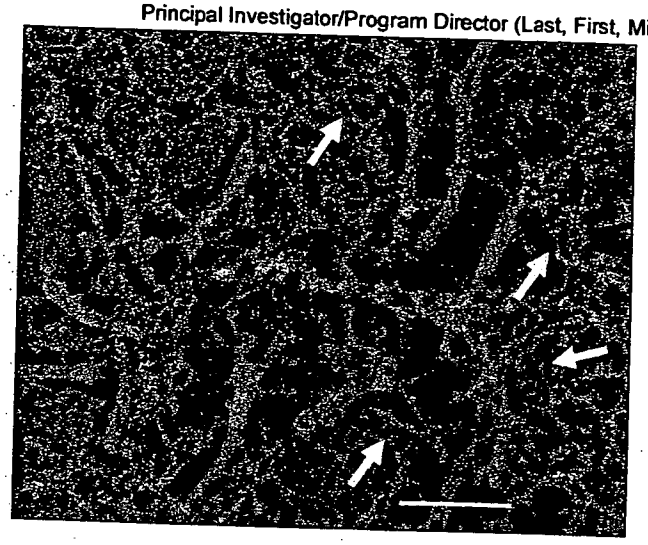


Figure 6. Scanning electron micrograph of *M. tuberculosis* H37Ra bacilli (red arrows) adhering to a glass substratum. Note the presence of a fibrillar meshwork (white arrows) tethering the bacteria, promoting bacterial interactions and the formation biofilm-like communities. Bar = 1 μm.

D. RESEARCH DESIGN AND METHODS.

General Methods

1. **A549 epithelial and U-937 macrophage-like cell lines.** A549 human type II alveolar epithelial cells and the human macrophage-like cell line U-937 were obtained from the American Type Culture Collection. A549 cells will be maintained in RPMI 1640 medium containing 10% heat-inactivated FCS (RPMI 1640-10% FCS), L-glutamine, non-essential amino acids, and sodium pyruvate. A549 cells will be seeded at 10^5 cells into 24-well tissue culture plates and allowed to adhere and grow to 80 or 100% confluence at 37°C in a CO_2 incubator before use in experiments. Suspension cultures of the human macrophage-like cell line U-937 will be maintained at 37°C in 5% CO_2 in RPMI 1640-10% FCS. For preparation of adherent U-937 monolayers in 24-well tissue culture plates, antibiotic-free cells will be treated with phorbol 12-myristate 13-acetate (25) as described in our published procedure (26). Viability of the tissue culture cells in the adherence/survival assays will be monitored by trypan blue exclusion (27) and adherent cell numbers will be counted by the naphthol blue-black method of Nakagawara and Nathan (28). Our tissue culture cells are routinely tested for the presence of mycoplasma.

2. **Human monocytes**. Human monocytes will be isolated from heparinized venous blood by Ficoll-Hypaque gradient centrifugation (27) and further purified using fibronectin adherence on gelatin coated flasks (133). Adherent monocytes will be recovered and added to 24-well tissue culture plates at a concentration of 2×10^5 monocytes/well in 1640 media containing 10% autologous human serum. Monocytes will be incubated at 37°C in a CO_2 incubator for 2 to 7 days before use in various assays. Purity of the monocyte preparation will be determined by Wright stain, and viability will be determined by trypan blue exclusion.

3. **Bacteria.** Mycobacterial strains (*M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. tuberculosis* CDC1551, *M. bovis* BCG) and *E. coli* strains (to be used in cloning of *M. tuberculosis* genes) will be stored as stock cultures at –70°C as thick suspensions in either Middlebrook 7H9 [mycobacteria] or LB [*E. coli*] medium containing 30% glycerol. Mycobacterial inocula for various experiments will be prepared by spreading several drops of a freshly thawed bacterial suspension to Middlebrook 7H10 agar plates which will be incubated at 37°C [three weeks for *M. tuberculosis*, three days for *M. smegmatis*] (9). Liquid cultures will be grown in Middlebrook 7H9 medium. For growth of *M. tuberculosis* H37Rv, H37Ra, and CDC1551 bacteriological media (plates and liquid) will include 10% oleic acid-albumin-dextrose-catalase supplement (OADC) and 0.05% Tween-80 in liquid cultures only (90). The bacilli will be harvested and diluted to appropriate concentrations for use in the various studies, following procedures described in the Preliminary Studies Section. Purity of cultures will be monitored by both acid-fast and Gram staining and by culturing on blood agar plates.

4. **Safety Precautions. Proposed work with virulent *M. tuberculosis* H37Rv and CDC1551 will be done in Dr. Friedman's BSL-3 (Biosafety Level 3) containment facility at the University of Arizona, College of Medicine. Work with virulent *M. tuberculosis* and infection of mice will be done in our newly completed BSL-3 animal laboratory in the Central Animal Facility, at the College of Medicine.** Experiments to clone *M. tuberculosis* DNA into a non-pathogenic mycobacterial host have been reported to the Biosafety Committee at the University of Arizona and have been assessed as requiring containment level 2. All infectious material will be handled in a biological safety cabinet. Safety precautions used and

recommended by the Centers for Disease Control (CDC) will be employed on this project for handling of bacterial samples.

5. **Statistical Analysis.** The significance of differences between results will be calculated by the analysis of variance (ANOVA). All significant differences between groups identified by two-way ANOVAs at the 95% confidence level or greater will be confirmed by post-hoc testing.

SPECIFIC AIM No. 1: To Characterize the Biochemical Properties of Mtp.

Background/rationale. There are no reports on the presence of pili produced by *M. tuberculosis* or any other mycobacterial species. As shown in the Preliminary Studies Section, several lines of evidence demonstrate that *M. tuberculosis* produces pili structures. Generally, pili are recognized as virulence factors since their presence enhances the ability of bacteria to adhere, colonize, and even invade epithelial cells (100). They are also potential vaccine candidates for inducing protective antibodies to block adherence and colonization (102). Mutants defective in production of pili are generally non-adhesive and less virulent than the parent strains. Under these premises, it is hypothesized that Mtp are likely to be important in the pathogenesis of *M. tuberculosis*. As described in Preliminary Studies Section, the Mtp pili did not dissociate into pilin monomers under conditions conventionally employed for denaturation and biochemical characterization of several pili types such as type I pili, type IV pili, curli, and the repertoire of colonization factors of enterotoxigenic and uropathogenic *E. coli* strains. Thus, other methods will be used to identify the amino acid sequence of the Mtp pilin monomer. This will would allow us to identify, map, and manipulate the genetic determinants involved in pili biogenesis (**see Specific Aim No. 2**). To this purpose, we will use the genome sequence database available for *M. tuberculosis* (29, 30). **Our collaborator, Frederick Cassels, Ph.D., Walter Reed Army Institute, will assist us in these studies. He has extensive experience in the biochemical characterization and study of a number of intractable bacterial pili (153-155). Please see his attached letter of collaboration and Biographical Sketch.**

1. **Determine amino acid sequence of Mtp pili.** As has been previously shown by Cassels and associates, the combination of liquid chromatography-mass spectrometry (LC-MS, Agilent 1100 MSD mass spectrometer) and LC-tandem mass spectrometry (LC-MS/MS, Q ToF II, Waters/Micromass) can be particularly useful in identifying fimbrial proteins or peptide fragments (176, 177). For pilus proteins that are not easily digested, Hess *et al.* (176) has developed a combination of chemical and enzymatic cleavages that allowed the characterization of more than 95% of *Actinomyces naeslundii* pili that were believed to be indigestible (176). Therefore, our collaborator Dr. Cassels is well-equipped and able to characterize the Mtp fimbriae by a similar approach. A Q ToF II mass spectrometer will be used to analyze the peptide fragments generated from a testing series of chemical and enzymatic cleavages. The cleavages to be considered will include but are not limited to cyanogen bromide (CNBr) cleavage, dilute acid hydrolysis, and the proteolytic enzymes Lys-C, trypsin, pepsin, Glu-C, Arg-C, and proteinase K. Given the nature of the pilin proteins, it is expected to be determined empirically which method works best with Mtp fimbria.

The Q ToF II mass spectrometer is a state-of-the-art hybrid quadruple time of flight mass spectrometer that enables one to determine amino acid sequences of known genes with high accuracy. In general, both in-house ProteinLynx Global Server 2.0 and MASCOT Web Server searches will be used to identify the peptides and reassemble them *in silico* to the respective Mtp proteins. **The increased sensitivity of LC-MS/MS over Edman degradation methodology offers improved opportunity to identify internal peptide sequence. This is possible because the sensitivity of Edman degradation for peptides and proteins is in the 50-100 pmol range, but LC-MS/MS is in the 1 pmol range.** When previous treatments followed by Edman degradation have not resulted in obtaining protein sequence, minor fragments not detected by Edman would readily be analyzed by LC-MS/MS. During FY04 of the grant a Thermo Finnigan LTQ with a sensitivity in the fentomol range will be installed at Walter Reed Army Institute, with improvements that will enhance the identification of unknown modifications.

Mtp samples will be run on 3-8% Tris-acetate polyacrylamide gels (able to resolve proteins of one million Da or greater)[Invitrogen, Carlsbad, CA] and stained for evidence of the ability to resolve in a large pore gel. All stained bands will be reduced and alkylated, trypsin treated, and peptides extracted for LC-MS/MS sequence analysis (178).

2. **Production of affinity-purified antibody against Mtp.** To characterize Mtp and its potential function in *M. tuberculosis* pathogenesis, affinity-purified rabbit antibodies against Mtp will be produced. Anti-Mtp antibody will be a useful tool in determining the role of Mtp in the interaction of *M. tuberculosis* with cultured host cells. The ability of anti-Mtp antibody to block bacterial adherence and/or invasion of host cells will be evaluated in inhibition of adherence assays as described in Specific Aim No. 3. Further, anti-Mtp antibody will also be used to monitor Mtp expression in clones and isogenic mutants to be constructed in Specific Aim No. 2. Antisera will be produced for these studies by Quality Controlled Biochemicals (QCB), Inc., Hopkinton, MA. Antibodies against Mtp (**purified as described in Preliminary Studies**) will be raised in New Zealand rabbits by subcutaneous and intramuscular injections of purified Mtp (150 µg/dose) with complete Freund's adjuvant in the first dose and with incomplete Freund's adjuvant in the following doses. Rabbits (two total) will be boosted five times and test bleeds will be taken at each stage to monitor antibody titers against Mtp by ELISA performed as described in the Preliminary Studies Section. The presence of specific antibodies will also be tested by IF microscopy as described in Preliminary Studies. Once a means is devised to denature Mtp into its pilin monomers or when a His-tagged-MtpA protein is produced in *E. coli* (**see Specific Aim No. 2, section 2b below**), Western blot analysis will also be used to test the antisera. Antibodies generated against Mtp will then be affinity-purified. In brief, rabbit serum will be passed over an agarose-based column prepared with either purified Mtp or Mtp pilin monomers chemically bound to it. The column will be washed with PBS and bound antibodies will be eluted using a low pH glycine buffer before dialysis in PBS. Alternatively, if problems arise with successfully binding Mtp to the agarose resin, then antibody will be purified by batch absorption using purified Mtp. Rabbit serum will be mixed with Mtp for several hours or overnight and Mtp with bound antibody will be recovered by ultracentrifugation, since in our initial studies we observed that Mtp can be isolated by high speed centrifugation. The bound antibodies will then be eluted using low pH buffer as described above. These affinity-purified antibodies will be stored frozen at –70°C until use.

3. **Production of Antibodies against His-tagged MtpA pilin and Mtp-derived peptides.** Once we have identified the open reading frame encoding the structural gene of the Mtp pilin monomer (*mtpA*) in the *M. tuberculosis* genome sequence, some interesting studies can be initiated. The *mtpA* gene will be subcloned into a His-tagged expression vector and will be used to overexpress MtpA pilin in *E. coli* and purified by nickel-agarose chromatography. **Please see Specific Aim No. 2, section 2b for details.** Using the deduced amino acid sequence of *mtpA* we will also be able to select candidate immunogenic regions and potential homologous host cell binding sites of the MtpA molecule for designing peptides. We estimate that five to ten peptides would be designed for this purpose. The immunogenic regions of MtpA will be determined and mapped using the HLA Peptide Binding Prediction software available on the NIH website (http:// bimas. dcrt.nih.gov/ molbio/hla_bind/) and possible host cell binding sites will be determined. Then Mtp 16-mer peptides to these selected regions will be synthesized to 95% purity by the University of Arizona Biotechnology Center, Tucson, AZ. The peptides conjugated to the carrier protein KLH by a cross linker, and His-tagged-MtpA will be used to immunize rabbits, and antisera will then be affinity-purified as described above. These anti-MtpA pilin and anti-MtpA-peptide antibodies will be used in inhibition of adherence experiments in Specific Aim No. 3. Other studies will also be done to determine the important epitopes of Mtp that are required for binding to host cells as well as in determining what regions carry immunogenic epitopes on the MtpA molecule.

The MtpA pilin monomer and peptides will also be used to determine their immunologic reactivity with sera from tuberculosis patients. These studies will be done using sera from TB patients (a total of 36) that we have already obtained from our colleague in Mexico (**Please Preliminary Studies and Fig. 4 for details**) and sera from 20 healthy controls will be collected. ELISA assays will be done with Mtp, pilin, and MtpA peptides to determine which proteins are reactive with the sera (156). In brief, ELISA plates will be coated by drying down the MtpA peptides, Mtp or pilin (1.25 µg/ml) onto wells, washed, and blocked with SuperBlock (Pierce). Antibody titers will be determined via ELISA assays following procedures described in the Preliminary Studies Section. Levels of total IgG as well as levels of IgG1 and IgG2a isotypes will be measured to determine whether Mtp induce either a Th1 or Th2-type immune response. Induction of a strong IgG2a response is ndicative of a Th1-type immune response, while a dominant IgG1 response is usually associated with a Th2-ype immune response (129). While we know that 60% of the TB sera are reactive with native Mtp (**by IF and**

ELISA, See Fig. 3 and 4), it will be interesting to determine if these same sera are reactive with the Mtp pilin monomer or its derived peptides. It is predicted that the patient sera will give positive ELISA results using the Mtp monomer and some of the Mtp peptides. These results will help to determine the key immunogenic epitopes on Mtp that induce a humoral immune response during a human tuberculosis infection. It is possible that one or more of these peptides may possess protective epitopes.

SPECIFIC AIM No. 2. To Clone the *mtpA* Gene and Construct Mtp-deficient Mutants.

Background/Rationale. The biogenesis of pili in general involves the participation of several genes specialized in defined functions such as processing, translocation, export, and assembly. These genetic determinants may be located either in the chromosome or on virulence plasmids. For example, while 14 clustered plasmid genes are needed for the production of the bundle-forming pilus (Bfp) by enteropathogenic *E. coli* (EPEC), around 30 widely distributed genes in the chromosome of *P. aeruginosa* are involved in biogenesis of type IV pili (56, 99, 101). Researchers have been able to clone and express type IV pili in *E. coli* K-12 and in heterologous backgrounds. To begin our studies of Mtp biogenesis, we will follow established strategies employed by those who have elucidated the role of pili genes in the biogenesis of the Pap, type I, and type IV pili (37, 63). Through a combined use of DNA cloning, detailed nucleotide sequencing and analysis, we hope to map and define the putative genetic determinants required for assembly of Mtp. **Importantly, we want to identify the Mtp structural gene *mtpA***. Further identification of Mtp-related genes by nucleotide sequence analysis will help determine the operon involved in the biogenesis of this pilus. Once *mtpA* is cloned, we will also construct a His-tagged-MtpA expression vector to produce the pilin monomer in *E. coli*. MtpA pilin monomer would be very useful in many of the proposed studies in this grant application.

1. **Strategies for cloning the Mtp gene if the pilin amino acid sequence is determined.** The identification of the primary amino acid sequence of the Mtp pilin will be directly obtained from the amino-, carboxy-, or internal amino acid sequence as determined in Specific Aim No. 1. The amino acid sequence will be used to identify the *mtpA* pilin structural gene in the *M. tuberculosis* H37Rv genome database (29). The complete deduced amino acid sequence of MtpA will also be used in searches of the GenBank-EMBL protein data base utilizing the BLASTP program to look for protein sequence homology of MtpA to any known bacterial proteins, or potentially, any previously identified pilin proteins. In most cases genes required for pili biogenesis are found to be located upstream or downstream from the structural pilin gene on the chromosome. Therefore, the region of the *M. tuberculosis* genome where *mtpA* is located will be analyzed for the presence of homologs of other genes typically involved in pili biogenesis. These include genes for prepilin peptidase, chaperones, minor pili subunits, proteins for membrane translocation, nucleotide binding proteins, and others. Predicted proteins of open reading frames flanking *mtpA* will be compared to protein sequences deposited in the GenBank using the NCBI search program.

As stated in the Background Section, it has been reported that *M. tuberculosis* contains a set of genes which are homologs of the *tad-flp* locus of *A. actinomycetemcomitans* that expresses type IV pili, with *flp1* being the pilin gene (130, 132). However, based on our preliminary results we are predicting that Mtp and Flp are unrelated since they are morphologically and biochemically distinct (88, 89, 132 and Preliminary Studies Section). If the Mtp amino acid sequence shows it to be a homolog of Flp, this will strengthen our notion that Mtp is indeed a pilus structure. If on the other hand, Mtp and the *M. tuberculosis* Flp-homolog are distinct this would suggest that *M. tuberculosis* may have more than one pilus operon. If Mtp and Flp show amino acid homology, then the *flp* gene will also be cloned, allelic exchange mutants constructed, and a complemented mutant strain made as described below in section 2 for constructing pili-deficient mutants.

In either case, DNA amplification by PCR, employing specific primers derived from the nucleotide sequence available in the *M. tuberculosis* genome database, will be performed to clone individually or in combination the set of genes with homology to other pilin sequences. The PCR products will be cloned into the shuttle vector pOLYG (137) using standard molecular biology techniques. pOLYG is a derivative of the hygromycin resistance vector p16R1, containing a multiple cloning site from pBluescript inserted into its *Kpn*I site (137). This vector can replicate in both mycobacteria and *E. coli* due to the presence of an origin of replication for each microbe (137). The pOLYG-*mtpA* gene construct and other constructs containing homologs of pili genes potentially involved in biogenesis will be transformed into *E. coli* DH5α, plated on LB agar plates containing 200 µg/ml hygromycin B and incubated overnight at 37°C. The plasmids will be

isolated from recombinant clones and the DNA inserts sequenced to verify no errors were incorporated into the DNA during PCR amplification.

Alternatively, to isolate DNA fragments from *M. tuberculosis* that may contain multiple genes of a potential Mtp operon, an *M. tuberculosis* H37Rv genomic DNA library already constructed in pOLYG and transformed in *M. smegmatis* 1-2c will be used (26). This library contains DNA inserts with an average size of 5.4 kb and was previously used to identify, isolate and characterize the *eis* gene of *M. tuberculosis* (26). Another *M. tuberculosis* genomic plasmid library containing larger DNA inserts of 10- to 12-kb will be constructed using pOLYG, as previously described (26), in case the operon might be contained on larger DNA regions. *M. smegmatis* containing the *M. tuberculosis* plasmid libraries will be plated on 7H10 agar plates containing hygromycin B and colonies will be screened by colony blot hybridization using a PCR-generated digoxigenen (DIG)-labeled probe for *mtpA* following standard protocols (26). *M. smegmatis* containing pOLYG-*mtpA* will be used as a positive control. It is anticipated that ten to twenty clones will be identified using this method that will contain *mtpA* and different 5' and 3' flanking regions of *M. tuberculosis* DNA. These plasmids will be isolated and characterized by restriction digest analysis to define the DNA regions that they contain. Insert DNA of unique clones will be sequenced, and the sequence will be subjected to BLAST alignment to the *M. tuberculosis* genome database in TubercuList (29). Using this method, it is anticipated that we will be able to identify the *mtpA* pilin structural gene and potentially other genes essential for pili biogenesis in *M. tuberculosis*.

2. Construction of an Mtp pili-deficient mutant in *M. tuberculosis*. A critical study to determine the role of Mtp as a potential virulence factor in *M. tuberculosis* is to construct a *mtpA*-deficient mutant. The mutant would then be tested, and compared to the parental wild-type and complemented strains, in adherence assays *in vitro* and in an aerosol mouse model for its ability to survive *in vivo* (**see studies proposed in Specific Aims No. 3 and 4**). These studies will be initiated by construction of an *mtpA* deficient mutant (Δ *mtpA*) in *M. tuberculosis* H37Rv and H37Ra. Using standard recombinant DNA methodology and known restriction map data on the *mtpA* gene of *M. tuberculosis*, a hygromycin resistance cassette will be inserted to inactivate the *mtpA* gene. This fragment will then be cloned into the allelic exchange vector pMJ10, a derivative of pPR27 (138), and named pMJ10/*mtpA-hyg*. The pMJ10/*mtpA-hyg* vector containing the inactivated *mtpA* gene will then be transformed into *E. coli* (139). Alternatively, in-frame deletions with non-polar effects will be constructed in the *mtpA* gene and cloned into pMJ10 (139). Thus, the *mtpA* gene will be inactivated without potentially causing polar effects on other downstream genes. Inactivation of the gene in the allelic exchange vector construct will be verified by restriction enzyme digestion analysis (139). The pMJ10/*mtpA-hyg* allelic exchange vector construct will be electroporated into electrocompetent *M. tuberculosis* H37Rv or H37Ra (26). Electroporated cells will be mixed with fresh 7H9 medium, and incubated at 37°C for 24 h. Bacterial cells will then be plated on appropriate selective media and incubated at 37°C for 3 to 4 weeks. Several single transformants will be grown separately in liquid media at 32°C and these will then be plated on 7H11/antibiotic plates containing 2% sucrose and incubated at 39°C to counterselect for the loss of the vector. Ten colonies of potential allelic exchange mutants will be picked after 3 to 4 weeks and PCR will be used to confirm that the chromosomal allele has been disrupted (139). Southern blot analysis will be done to verify the mutations. Potential allelic exchange mutants will be analyzed by TEM to confirm the loss of Mtp production. Colonies of potential mutants will also be screened by colony immunoblots to confirm loss of production of Mtp using affinity-purified anti-Mtp antibody. Once methods have been developed to denature native Mtp, lysates of potential allelic exchange mutants will be prepared and Western blot analysis done to confirm that no Mtp protein is produced. These are standard methods routinely used in Dr. Friedman's laboratory and it is anticipated that the pili mutant will be constructed without any problem.

2a. Complementation of *mtpA* deficient mutants. To complement the pili mutant, an integrating mycobacterial vector pMV306 (140) will be used to directly insert a wild-type copy of *mtpA* back into the chromosome of the *M. tuberculosis* mutant. We have been successful in using this integrative vector system previously to complement other allelic exchange mutants in our laboratory. pMV306 is based on mycobacteriophage L5 (141) and lacks a mycobacterial origin of replication (141). This plasmid carries the phage attachment site (*attP*), an integrase gene (*int*), a kanamycin resistance gene for selection of transformants, and a polylinker for insertion of DNA into the vector. Therefore, the vector can efficiently transform mycobacteria only via integration into a specific chromosomal site (*attB*) and is stable because the excisionase gene is not present for release of the inserted sequence (142). Using the integrative vector pMV306, we will insert a single copy of *mtpA* into the chromosome of the *M. tuberculosis* Δ *mtp* mutant.

Southern blot analysis will be done to confirm the presence of a single copy of the wild-type *mtpA* allele and the mutated allele containing the hygromycin gene in the complemented mutant. Both colony blot and Western blot analysis will be done on the complemented mutant to demonstrate production of the Mtp protein. TEM will also be done to confirm that the complemented mutant now produces Mtp.

2b. Construction and production of His-tagged-MtpA protein. Since presently we have been unable to denature native Mtp into its pilin monomers, the *mtpA* gene will be subcloned into a His-tagged expression vector system and overexpressed in *E. coli*. The availability of pilin protein would be useful in many of the proposed studies in this grant application. His-tagged-MtpA will be overexpressed by either using the vector pQE (Qiagen) or pET (Novagen) expression system following the manufactures recommend procedures. Bacterial cells will be harvested and disrupted by sonication. Total lysates will be batch absorbed to nickel-NTA agarose under denaturing conditions in a urea buffer. The nickel agarose will be washed, recovered by low speed centrifugation, and poured into a chromatographic column. After further washing, the His-tagged-MtpA protein will be eluted from the column with buffer containing imidazole, according to standard protocols. Eluted His-tagged-MtpA will be dialyzed against PBS to remove urea and imidazole. SDS-PAGE, Western blot analysis, and two-dimensional gel electrophoresis will be used to monitor purification of the MtpA protein. If problems occur using histidine tags, then other affinity tags are available (glutathione-S-transferase [GTS], maltose binding protein, thioredoxin, etc) and will be used.

3. Strategies to be considered for identifying and cloning Mtp genes if the pilin amino acid sequence is not determined. If methods to determine the amino acid sequence of Mtp are unsuccessful by standard protein sequencing techniques, then alternative methods described below will be used to identify the Mtp pilin structural gene.

3a. Construction of a transposon mutagenesis library in *M. tuberculosis*. A library of Tn*5367* transposon mutants will be generated in *M. tuberculosis* H37Rv following the protocol of Bardarov *et al.* (143). This approach uses a conditionally replicating vector that is able to replicate at 30°C but not at 37°C. Using this system Bardarov and associates have been able to generate transposon mutant libraries in *M. bovis* BCG and *M. tuberculosis* Erdman with efficient random insertion of Tn*5367* (143). The transposon mutant library will be grown on 7H11-OADC agar plates containing kanamycin (20 µg/ml) for selection of mutants containing transposon insertions. Colony immunoblot analysis will be done using affinity-purified anti-Mtp antibody, as previously described (157) to identify library clones that do not produce Mtp. Alternatively, if a specific erythrocyte type is found to be agglutinated by Mtp (**see proposed HA experiments in Specific Aim No.3, section 5 that follows**), then HA assays may be used to screen the transposon mutant library for clones that do not agglutinate these RBC. If problems arise in the construction of the Tn*5367* transposon library in *M. tuberculosis* H37Rv, we will then request the already constructed transposon mutagenesis library in *M. tuberculosis* Erdman from the laboratory of Bill Jacobs (143).

Transposon insertion into the putative pilin structural gene (*mtpA*), various pilin biogenesis genes, and possible regulatory genes would all result in a non-pilus phenotype. Thus, multiple Mtp negative mutants will be identified, isolated, and further characterized. TEM will also be used to verify that the mutants do not produce Mtp. To identify the location of the Tn*5367* insertion in the various mutants, genomic DNA will be isolated from 20 to 40 clones not producing Mtp as previously described (26). The gene inactivated by transposon insertion will be identified by inverse PCR, using the known transposon junction DNA sequences as primers (53). The DNA sequences obtained (400 to 600 bp) will be subjected to BLAST alignment to the *M. tuberculosis* sequence database in TubercuList (29) and also compared against DNA and protein sequences deposited in GenBank. Using these methods it is anticipated that we will be able to identify the *mtpA* pilin structural gene and potentially other genes essential for pili biogenesis in *M. tuberculosis*.

3b. Screening an *M. tuberculosis* H37Rv genomic DNA plasmid library in *M. smegmatis* for the production of Mtp. *M. smegmatis* is an ideal strain to use to express and produced Mtp in the proposed experiments. *M. smegmatis* is closely related to *M. tuberculosis* and has been demonstrated in various investigations to readily express genes from *M. tuberculosis* (144-147) and from other mycobacteria (148-150). Our Preliminary Studies have found that while *M. smegmatis* does produce pili, they are morphologically and antigenically different than Mtp. *M. tuberculosis* H37Rv genomic DNA libraries prepared in the vector pOLYG, as previously described (26) and **as discussed in Specific Aim**

No. 2, section 1 above, will be used in these experiments. *M. smegmatis* containing the *M. tuberculosis* plasmid libraries will be plated on 7H10 agar plates containing hygromycin B and colonies will be screened for the production of Mtp by colony immunoblotting using specific anti-Mtp antibody that will be pre-absorbed with *M. smegmatis* 1-2c cells to remove any cross-reactive antibodies. *M. smegmatis* clones that are positive by colony blotting for Mtp will be confirmed by TEM and IF as previously described (69). Morphologically, Mtp are curli-like in appearance while Smp appear as long rope-like bundles (**see Fig 1**). Thus the expression and production of *M. tuberculosis* pili by *M. smegmatis* should be readily detectable by using these techniques. Plasmids from Mtp-positive clones will be isolated and the insert DNA will be characterized by restriction digest analysis to define the DNA regions that they contain. DNA sequencing will be done on DNA inserts of ten to twenty unique clones, and the sequence obtained will be subjected to BLAST alignment to the *M. tuberculosis* sequence database in TubercuList (29). Using this method, it is anticipated that we will be able to identify the *mtpA* pilin structural gene and potentially other genes essential for pili biogenesis in *M. tuberculosis*.

At the present time it is not known if the genes for production of Mtp or Smp are homologous between *M. tuberculosis* and *M. smegmatis*, respectively, nor if each contains potential pili operons. If homologs for pili biogenesis genes are present in the *M. smegmatis* genome, then it would increase the likelihood that this microbe could produce Mtp when *mtpA* is present in *trans*. If such genes are not present and are required for production of Mtp then it would be less likely that Mtp would be produced by *M. smegmatis*. Once we have identified the *mtpA* gene and other potential Mtp biogenesis genes in the *M. tuberculosis* genome, searches of the *M. smegmatis* genome database at the website for the Institute for Genomic Research will be done to determine whether homologs may be present.

SPECIFIC AIM No. 3:To Define the Relationship Between Expression of Pili and the Ability of *M. tuberculosis* to Adhere, Enter, and Survive Intracelluarly within Host Cells.

Background/rationale. In this aim we are proposing to address specific questions regarding the biological function and role of Mtp in adherence and interaction of the bacteria with host epithelial cells and macrophages. Various research groups have demonstrated that *M. tuberculosis* has the ability to interact, invade and persist within both lung epithelial cells and macrophages (**see Background section for details**). Thus, studies will be done to determine whether inactivation of the pili structural gene (*mtpA*) will affect mycobacterial interactions with these host cell types. We will evaluate the capacity of *M. tuberculosis* H37Rv and H37Ra, pili deficient-mutants, and complemented mutants to adhere, invade, and survive within A549 epithelial cells, U-937 macrophages, and human macrophages. By using Mtp-expressing bacteria and isogenic mutants unable to express Mtp, we will be able to elucidate the contributive adhesive properties of Mtp in these processes. We will also study other biological properties associated with well-characterized pili such as bacterial aggregation, erythrocyte agglutination, binding to extracellular matrix proteins, and the ability of Mtp to stimulate production of pro-inflammatory molecules.

1. **Adherence and intracellular survival of *M. tuberculosis* and pili mutants in A549 cells, U-937 macrophages, and primary human monocytes**. We will evaluate the capacity of *M. tuberculosis* H37Rv and H37Ra, pili deficient-mutants, and complemented mutants to adhere, invade, and to survive within A549 epithelial cells, U-937 macrophages, and human macrophages. For optimal production of pili *M. tuberculosis* H37Ra will be grown on 7H11 agar plates containing OADC, while H37Rv will be grown in 7H9 broth containing OADC (**see Preliminary Studies for details**). The inoculum will be prepared by mixing bacteria into HBSS with gentle vortexing. Bacterial clumps will be disaggregated by the addition of sterile 3-mm glass beads followed by gentle vortexing. By this method we have been able to obtain highly dispersed *M. tuberculosis* inoculum that still contains pili. We have observed that removal of Mtp from the bacterial surface requires very high levels of mechanical shearing not achieved under the conditions used to prepare the inoculum. Bacterial preparations will be diluted in RPMI 1640 to appropriate concentrations and used in the various assays. Viable plate counts will be done on the initial inoculum used in all studies. Tissue culture monolayers will be infected using a multiplicity of infection (MOI) of 10 bacteria per cell (26). If this MOI is not high enough, we will use an MOI high enough to give countable CFU, but not so high as to cause host cell toxicity in the experiment. The bacteria will be incubated with the monolayers at 37°C in a CO_2 incubator for 1, 2, or 4 h before the wells are washed with tissue culture media. For experiments to monitor long-term intracellular survival, monolayers will be infected for 1, 2, or 4 h, treated as described below, and incubated for

up to 7 days. To differentiate between adherent *M. tuberculosis* and microbes that are intracellular, one set of wells will be treated with amikacin (200 µg/ml) for 1 h at 37°C to kill extracellular bacteria while another set of wells will not be treated with antibiotic. Amikacin at this concentration kills extracellular bacteria but has no effect on bacteria within the tissue culture cells (26, 38, 91). Monolayers will be washed several times with HBSS and at various time points lysed by the addition of one ml of 7H9 containing 1% Triton X-100. Serial dilutions of lysates will be spread on 7H11-OADC agar plates and incubated at 37°C for 3 weeks before viable plate counts for each strain are tabulated. Thus, the number of adherent extracellular bacteria at each time point will be calculated by subtracting antibiotic-treated well plate counts (CFU of total intracellular bacteria per well) from antibiotic-untreated well plate counts (CFU of both adherent and intracellular bacteria per well). Experimental controls of *M. tuberculosis* H37Rv or H37Ra and mutants incubated in RPMI-1640-10% FCS media alone over the same time periods will be done to verify that the microbe does not replicate in tissue culture media alone. Alternatively, methods will be used to block bacterial internalization and measure adherence. This will include incubation at 4°C or treatment with inhibitors of internalization such as cytochalasin D or monodanylcadaverine (175).

A flow cytometric analysis method will also be used to directly differentiate between attached and internalized *M. tuberculosis* interactions with host cells (163). This technique uses FITC-labeled bacillus and quenching by trypan blue of extracellular fluorescent bacteria to determine numbers of adherent and intracellular organisms in the assay. This is a method that we have previously used to study the interactions of *Bordetella pertussis* with PMN (163). Others have also used similar methods to monitor phagocytosis of yeast, *E. coli*, *S. aureus*, and *M. tuberculosis* (164-167). Briefly, *M. tuberculosis* wild-type, pili deficient, and complemented mutants at 10^8 bacteria/ml will be labeled with FITC by incubation in PBS for 30 min at 37°C, followed by centrifugation, and washing to remove residual unbound FITC. *E. coli* will also be FITC-labeled and used as a control in these studies. FITC-labeled bacteria will be added to host cells as described above and at various time points (30 min, 1, 2, 4, and 8 h) cells will be put on ice, monolayers washed, and tissue culture cells recovered by use of small glass beads. Samples with or without the addition of trypan blue will be analyzed by flow cytometric analysis using a Becton Dickinson flow cytometer (163). Fluorescence associated with host cells indicates total FITC-labeled bacteria both adhered and internalized. Addition of trypan blue quenches all extracellular, but not intracellular, fluorescence. Thus one can determine the percentage of adherent and intracellular *M. tuberculosis* associated with host cells by using this method.

To visually monitor the interaction of *M. tuberculosis* and the pili-deficient mutants with host cells, light and scanning electron microscopy (SEM) studies will also be done in conjunction with the above proposed studies. For light microscopy, monolayers will be prepared on glass cover slips in 24-well tissue culture plates. Host cells will be infected as described above and coverslips removed from wells before treatment with antibiotic. The coverslips will then be fixed in 3% formalin, mounted on glass slides using Permount, and then acid-fast stained followed by Giemsa counter-staining. Coverslips will then be observed under oil-immersion using a Nikon TE 2000S light/fluorescent microscope to look at the levels of total bacterial association with host cells at various time points in the experiments. To further confirm the presence of pili on adhering M. *tuberculosis*, utrastructural analysis will also be done using high resolution SEM of infected monolayers as previously described (104). Briefly, infected monolayers on glass coverslips will be fixed in 3% formalin, postfixed in 1% osmium tetraoxide, dehydrated in sequential ethanol concentrations, critically point dried, and coated with a mixture of gold and paladium. The specimens will be examined using a Phillips scanning electron microscope available for our use in the Arizona Laboratory, Division of Biotechnology Imaging Facility. As positive and negative controls for the presence of pili, wild-type and pili mutant *M. tuberculosis* preparations, as well as uninfected host cells will also be prepared. These SEM studies will provide visual evidence of *M. tuberculosis* fibers involved in adherence to host cells. If filamentous structures are observed, we will determine if these are Mtp by immunogold SEM. The presence of fibers on adhering *M. tuberculosis* would suggest a role in host cell colonization.

In these experiments it is expected that wild-type *M. tuberculosis* expressing pili will adhere better than the pili-deficient mutants and will likely more efficiently enter A549 cells, U-937 macrophages, and human blood-derived macrophages. Intracellular numbers and growth of the wild-type bacilli may be increased by virtue of higher numbers of bacilli adhering and entering these host cells.

2. **Binding of purified Mtp or pilin monomers to host cells**. We will also assess the ability of purified Mtp filaments, pilin monomers, or fluorescent-beads (0.5 um, Dynamics Corp., Portland, OR) coated with

these proteins and Mtp derived peptides to bind directly to cultured A549 cells, U-937 macrophages, or human macrophages. Mtp, pilin, or beads coated with these proteins, as indicated by the manufacturer's instructions, will be incubated with host cells (cultured on cover slips) for 4 h and then washed. Uncoated beads will be used as a control. The presence of fluorescent-beads will be observed directly by fluorescence microscopy, while bound pili or pilin will be detected by IF using affinity-purified anti-Mtp antibody followed by incubation with goat anti-rabbit IgG Alexa Fluor 488. Alternatively, Mtp fibers will be biotinylated with amino-sulfo-biotin (Pierce) and binding of the pili to host cells will be detected using streptavidin conjugated to Alexa Fluor 488 and fluorescence microscopy. These experiments will provide significant information regarding the direct role of Mtp in the interaction of *M. tuberculosis* with eukaryotic cells.

3. **Effect of specific antibody against Mtp, ManLAM, and HBHA on adherence and entry into host cells**. One problem that may arise in these investigations is the presence of other recognized *M. tuberculosis* adhesins, in particular ManLAM (32, 33) and the heparin-binding hemagglutinin adhesin [HBHA] (39-42). ManLAM plays a role in adherence of the microbe to macrophages via the mannose receptor, while HBHA is important in adherence to epithelial cells via surface sulfated glycoconjugates. To differentiate the adhesive role of Mtp to host cells from ManLAM and HBHA, experiments will be done using specific antibodies against these adhesins. Mouse monoclonal antibodies to ManLAM will be obtained from the NIH TB contract center at Colorado State University, and anti-HBHA mouse monoclonal antibodies will be obtained from Mike Brennan at the FDA (40). Anti-Mtp antibody preparations will be added singly or in combination with anti-ManLAM and anti-HBHA to the adherence assay, as described above, to determine what level of adherence to host cells is due to each adhesin. The effects of both anti-ManLAM and anti-HBHA will also be tested separately and in combination in the assay. Preimmune rabbit serum, normal mouse serum, and buffer alone will be used as controls. By comparing the decrease in levels of bacteria associated with host cells caused by each antibody preparation we will be able to determine the level of attachment conferred by each of the mycobacterial adhesins. **It is anticipated that these experiments will help to differentiate the role of pili from known *M. tuberculosis* adhesins in the attachment to host cells and will potentially show that Mtp do play a role in adherence.**

4. **Role of *M. tuberculosis* pili in bacterial aggregation.** It is well known that *M. tuberculosis* and other mycobacterial species when grown *in vitro* will readily form bacterial aggregates. It is has been reported that adhesins of other bacterial pathogens can cause bacterial aggregation or autoaggregation (92, 93). Therefore, studies will be done to determine if Mtp may play a role in *M. tuberculosis* aggregation. Wild-type *M. tuberculosis* H37Rv, its isogenic pili mutant, and complemented strain, will be grown on 7H11 + glycerol agar plates for production of Mtp as described in the Preliminary Studies Section. Bacteria from plates will be suspended as single cell suspensions in PBS to an OD_{600} of 1.0. Then 10-ml aliquots will be removed for each strain and placed into 15-ml conical tubes and the bacterial suspension allowed to settle at room temperature. Microtiter plate assays will be done to quantitatively monitor levels of bacterial aggregation (clumping) over time. Two hundred microliter samples of supernatant will be removed from the top of each tube at 15 min intervals, over a 4 h time period, and added to flat-bottom microtiter plates. Turbidity readings of the removed supernatant samples will measured at OD_{600} using a microtiter plate reader. With increased aggregation of the bacteria (wild-type and complemented strain) the turbidity readings will decrease over time as compared to non-clumping bacteria (pili mutant) where absorbance readings should remain constant or only slightly decrease. If the pili mutant demonstrates lower levels of aggregation, various concentrations of purified Mtp will be added to the mutant to determine if this would complement the decrease in aggregation. If this is the case then one should observe a dose-dependent increase of pili mutant aggregation with the addition of increasing amounts of Mtp.

5. **Agglutination of erythrocytes by *M. tuberculosis* pili**. A classic biological activity of many bacterial pili is their ability to agglutinate erythrocytes (117). Hemagglutination (HA) in many cases correlates with production of pili, adherence, and virulence (121, 130). Some examples include ETEC, UPEC, *Bordetella*, and some viruses. HA assays are also used as a standard method to study lectin-like attachment of bacteria to host cell receptors. Thus, studies will be performed to screen the ability of purified Mtp to agglutinate erythrocytes (RBC) from various types of animals. The RBC types to be tested in HA assays will include guinea pig, chicken, cow, horse, goose, rabbit, mouse, rat, turkey, sheep, goat, and human type A, B, O (Rh- and Rh+) erythrocytes. HA assays will be performed at 4, 26, and 37°C since it has been observed that some

pili types only agglutinate RBC at specific temperatures (130). By screening various types of RBC it is anticipated that we will find a specific erythrocyte type that will be agglutinated by Mtp in a dose-dependent manner. To verify that the observed HA is due to Mtp, experiments will also be performed in the presence of anti-Mtp antibody which should inhibit the HA activity. Preimmue rabbit sera will be used as a control in these studies. Once a specific type of RBC is identified that hemagglutinates with Mtp, studies will be done to identify a putative host cell receptor on RBC using various soluble carbohydrate compounds to inhibit hemagglutination activity. These will include sugars such as mannose, glucose, galactose, dextran, various sulfated carbohydrates (fetuin, dextran sulfate, chondrortin sulfate, heparin and fucoidan) as well as *N*-acetyl-glucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, *N*-acetylneuraminlactose, and galactose α-(1-4)-galactose (130). Blockage of Mtp HA by addition of sugars would be evidence that pili are binding to host cell carbohydrate receptors.

6. **Interactions of extracellular matrix proteins with *M. tuberculosis* pili.** Studies presented in the Preliminary Studies Section (**see Fig. 5**) demonstrate that Mtp binds to ECM, in particular to laminin in a dose-dependent manner. Laminin-binding proteins have been reported by others to be produced by *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (43-45, 172). Our results strongly suggest that Mtp may act as a *M. tuberculosis* adhesin by allowing the microbe to attach to lung ECM to initiate adherence and colonization. Further studies on Mtp binding to ECM will be done to confirm and extend these initial experiments.

Wells of microtiter plates will be coated with purified Mtp, His-tagged-MtpA pilin monomer, and Mtp peptides and blocked using Superblock (Pierce) as described in the Preliminary Studies Section. Various human or mouse ECM will be added to coated wells including laminin, fibronectin, collagen IV, and virotnectin over a protein concentration of 0.01 µg/ml to 10 µg/ml per well. BSA will be added to wells as a negative control. ELISA plates will be incubated, washed, and bound ECM will be detected using specific anti-ECM antibodies (Sigma) and peroxidase conjugates as previously described. The ELISA assays will be read at OD_{450} using a microtiter plate reader. These experiments will reconfirm our initial studies that Mtp binds to laminin and fibronectin and does not bind to collagen IV and will determine if Mtp binds to vitronectin. Additionally, these studies will also determine whether ECM will bind to native Mtp, MtpA pilin monomer, as well as to Mtp peptides. By performing binding assays using Mtp peptides we may be able to determine which regions of Mtp protein are involved in adherence to ECM. To verify specificity of Mtp binding to ECM, anti-Mtp antibody, anti-MptA pilin, and anti-Mtp peptide antibodies will be used in the ELISA assays to block Mtp and Mtp protein derivatives from binding to ECM.

Assays to study the adherence of *M. tuberculosis* bacteria to ECM will also be done using the matrix proteins that are found to bind to Mtp by ELISA assays as described above. This bacterial adherence assay will be done following the method of Fink *et al.* (173). Twenty-four well tissue culture plates will be coated with ECM at 10 and 50 µg/ml. As controls, some wells will be untreated and others coated with BSA. Wild-type *M. tuberculosis*, pili mutant, and the complemented strains, all at 10^7 bacteria per ml in HBSS, will be added to ECM-coated wells and incubated at 37°C in a humidified CO_2 incubator for 1, 2 and 4 hWells will then be washed with HBSS at the various time points, bound bacteria will be recovered by treatment with one ml of 7H9 containing 1% Triton X-100, and viable plate counts will be determined. The percent adherence will be calculated by dividing the number of adherent CFU per well by the number of inoculated CFU. If Mtp play a role in binding to ECM, the pili mutant should bind at lower numbers than the parental or the complemented strain. To demonstrate the role of Mtp in binding to ECM in the assay, experiments will also be done using anti-Mtp antibody. If Mtp plays a role in binding to ECM, the presence of anti-Mtp in the assay will decrease the adherence of wild-type bacteria and the complemented strain to the ECM–coated wells. Alternatively, if problems arise with this methodology, ELISA plates coated with *M. tuberculosis* will be used to monitor binding of ECM as described by Marques *et al.* (172).

We anticipate that these proposed studies will confirm and extend the evidence that Mtp binds to laminin and fibronectin and perhaps other ECM. Such results would suggest that binding of *M. tuberculosis* to ECM via Mtp might play a critical role in the microbes' ability to colonize and possibly disseminate within the human host.

7. **Do *M. tuberculosis* pili stimulate production of pro-inflammatory cytokines?** In human tuberculosis the host mounts a cell-mediated immune response to the infection which leads to the recruitment and activation of macrophages and T cells (122). The microbes' ability to survive this response culminates in the development of a granulomatous lesion to contain and wall off the bacterial infection (122). Bacterial-host cell

interactions lead to the induction and release of various proinflammatory mediators by both macrophages and resident epithelial cells in response to the infection. It has been reported that some bacterial products including LPS, flagella, and pili from different microbial pathogens induce secretion of a variety of proinflammatory cytokines (123, 124,161,162). We are interested in determining if Mtp causes release of proinflammatory cytokines by both human macrophages and A549 alveolar epithelial cells in response to *M. tuberculosis* pili. Previous studies by Bermudez *et al.* reported that after infection with *M. tuberculosis*, A549 cells were stimulated to secrete various cytokines (38).

Monolayers of both cell types will be prepared in 24-well tissue culture plates as previously described and various concentrations of purified Mtp (1, 10, 50, and 100 µg/ml) will be added to the monolayers for 1, 2, 4, 8, 16, or 24 h. Cell culture supernatant will be isolated at these time points and frozen at -70°C until use for determination of cytokine levels. Secretion of cytokines IL-1α, IL-6, and IL-8 will be determined using ELISA sandwich immunosorbent assays (R & D Systems or Biosource International). The cytokine bead array technique (BD Bioscience) will be used to evaluate the presence of cytokines IFN-γ, TNF-α, IL-2, 4, 5, and 10 simultaneously (125). The cytokine bead array assay will be performed according to the manufacturer's instructions. Briefly, antibodies against each of the above cytokines are provided coated onto six different groups of beads that differ in their fluorescence intensity. The beads are mixed together with a second group of cytokine specific antibodies conjugated to phycoerythrin. Supernatants from the pili-treated macrophages and A549 cells will be added to the bead antibody mixture and incubated for 3 hours at room temperature. The beads will be washed and analyzed by flow cytometry using a BD FacScan flow cytometer, which is part of the core facilities at the University of Arizona, College of Medicine. Concentrations of each cytokine will be determined based on known amounts of standard cytokine added to a set of control beads. Monolayers incubated with buffer only and host cells treated with PPD (Mycos Research) at 1 and 10 µg/ml, will be used as negative and positive controls, respectively. It is anticipated that Mtp will influence the production of proinflammatory cytokines by both human macrophages and A549 epithelial cells. If positive results are obtained using Mtp then the ability of wild-type *M. tuberculosis* and the pili mutant will be tested in the assay to determine if a difference in cytokine release is observed. Such results would suggest that Mtp potentially could be an early antigen sensed by the host to respond to and modulate the immune response to a tuberculosis infection.

SPECIFIC AIM No. 4: To Investigate the Effect of Pili Gene Inactivation on Survival and Multiplication of *M. tuberculosis* in Mice and to Evaluate the Protective Efficacy of a Mtp Vaccine.

Background/rationale. To determine the possible role of Mtp *in vivo*, the ability of the pili mutant to colonize, persist, and replicate as compared to wild-type H37Rv will be analyzed using a mouse respiratory aerosol model (126-128). **A decreased ability of the pili deficient mutant to colonize and survive in mice would strengthen our hypothesis for the role of Mtp in tuberculosis pathogenesis.** Studies will also be done to investigate the potential protective properties of Mtp against tuberculosis infections in mice.

1. **Effect of pili gene inactivation on survival and multiplication of *M. tuberculosis* in an aerosol mouse model.** Eight week old, specific-pathogen-free female C57BL/6 mice will be used in these studies. Animals will be maintained under barrier conditions and fed commercial mouse chow and water *ad libitum*. *M. tuberculosis* H37Rv, the pili mutant, and complemented mutant will be grown as previously described for optimal production of pili and used as inoculum for these animal studies. Mice will be challenged with *M. tuberculosis* via the aerosol route using a bacterial suspension that will introduce 50 to 100 bacilli into the mouse lungs over a 30 min exposure period using a Middlebrook chamber [Glas-Col, Terra Haute, IN] (127). **The aerosol chamber and tissue homogenizer for these studies will be purchased for the proposed work using funds from this grant.** This work will be done in our newly completed Biosafety Level-3 animal laboratory in the Animal Care Facility, at the University of Arizona College of Medicine. **Sheldon L. Morris, Ph.D., Chief of the Laboratory of Mycobacterial Diseases and Cellular Immunology, at the Center for Biologics Evaluation and Research, FDA, in Bethesda, MD will be a collaborator for these animal studies. Please see his Biographical Sketch and attached letter of collaboration. Dr. Morris has extensive experience using this aerosol infection system to study the efficacy of new tuberculosis vaccines in mice.** He will help in the design of the animal studies and assist in the training of Dr. Friedman and others from his research group in the use of the Middlebrook aerosol chamber.

A total of 50 mice per study group will be infected. At various time points (24 h, 2, 4, 6, 8, 10, 12, 16, 20, and 32 weeks post-infection) five mice per group will be euthanized and the lungs and spleen will be aseptically removed. A group of mice will be sacrificed at 24 h after infection to verify the size of the aerosol challenge dose. The left lower lobe of each lung will be removed and fixed in 10% neutral buffered formaldehyde for histological analysis (**see below**). The rest of the lungs and the spleens will be separately homogenized in 5 ml of 0.04% Tween 80-PBS using a Seward Stomacher 80 homogenizer (127). Organ homogenates will be diluted and plated on 7H11-OADC agar plates, using appropriate antibiotics, for viable plate count determinations. **It is expected that the pili mutant will exhibit decreased ability to colonize the lung, to persist and multiply in mice as compared the virulent *M. tuberculosis* H37Rv strain.** The complemented mutant should give similar results as the wild-type parent H37Rv. It is also possible that Mtp may play a role in initial lung colonization and in systemic spread. If pili play such a role then lower numbers of the pili mutant may be detected in the spleen of infected animals as compared to the wild-type parent.

For histological analysis, fixed mouse lungs will be embedded in paraffin, sectioned, and stained with either hematoxylin/eosin or Ziehl-Neelson stain and evaluated by light microscopy. This will be done by the Arizona Veterinary Diagnostic Laboratory, at the University of Arizona. Some tissue sections will also be used for immunofluorescence assays or in immunogold TEM using anti-Mtp antibody to determine directly if the bacteria are producing pili during infection of the mice. These studies will allow us to assess the level of lung pathology present at the different time points after infection and compare the degree of lung damage, inflammation and development of granulomatous lesions between H37Rv,the pili mutant, and the complemented strain. Histological analysis, using the acid-fast stain, will allow us to verify that viable plate count data is consistent with observed levels of acid-fast bacteria present in lung tissue. If the pili mutant is less able to colonize and replicate within the lungs, then lower numbers of acid-fast bacilli will be present and observed pathology should be decreased and directly proportional to decreased viable plate counts.

2. <u>Evaluation of the protective efficacy of *M. tuberculosis* pili against aerosol challenge.</u> Survival studies will be done to evaluate whether Mtp can stimulate a protective immune response in mice against a *M. tuberculosis* aerosol challenge. Groups of C57BL/6 mice (10 mice each) will be immunized via the subcutaneous route with purified Mtp at 10, 50 or 100 µg in Freunds incomplete adjuvant and then given two additional boosters at the same antigen concentration at two week intervals. After Mtp immunization, sera will be obtained from mice and tested in ELISA for the presence of anti-Mtp titers to verify that the vaccination regimen has stimulated an immune response in the animals. If need be, animals will be immunized longer to induce at least a strong humoral immune response. As a positive control, a group of mice will be vaccinated subcutaneously with 5 X 10^6 CFU of *M. bovis* BCG Pasteur (127, 128), while a negative control group will only be treated with buffer injections. Mtp-vaccinated mice will be challenged by the aerosol route, two weeks after receiving their final booster, while mice vaccinated with BCG will be infected six weeks after immunization. Mice will be infected with 50 to 100 CFU of *M. tuberculosis* H37Rv via the aerosol route using a Middlebrook chamber as described above. Mice will be maintained until they become moribund and then they will be euthanized. Survival of vaccinated and control mice will be monitored and tabulated over a 400 day period. If the *M. tuberculosis* pili stimulate a protective immune response in mice, then one should observe a significant extension of survival time as compared to the unimmunized control mice. It will be interesting to compare the survival rate of the Mtp vaccine group as compared to the BCG vaccine group, since BCG is a standard vaccine that gives excellent protection in mice against an *M. tuberculosis* aerosol challenge (127, 128).

2a. <u>Evaluation of the immune response to Mtp vaccination.</u> If Mtp vaccination is found to significantly protect mice against an aerosol TB infection, then further studies will be done to investigate the protective immune response that was induced by this antigen. To do this, the humoral and cellular immune responses in Mtp-vaccinated mice and a vaccinated group challenged with TB will be studied. **Dr. Emmanuel Akporiaye, an immunologist in the Department of Microbiology and Immunology, University of Arizona, College of Medicine will be a collaborator in these mouse immunology studies. He has extensive experience studying the cell-mediate immune response of mice in response to tumors and has direct experience with the procedures that will be used. Please see Dr. Akporiaye's attached letter of collaboration and Biographical Sketch.**

Mice will be vaccinated with the optimal dose of Mtp as determined in the protection studies described above. Control mice will be given buffer only, while another group of mice will be immunized with BCG and used as a positive control in these studies. These three groups of mice will then be sacrificed 30 days after their last vaccination. Another set of vaccinated animals will be aerosol challenged with *M. tuberculosis* H37Rv

and sacrificed 5 and 6 weeks later. Before death, blood will be recovered via bleeding from the orbital plexus of mice and sera will be isolated. Anti-Mtp antibody titers will be determined via ELISA assays as described above. Levels of total IgG as well as levels of IgG1 and IgG2a isotypes will be measured to determine whether Mtp induces either a Th1 or Th2-type immune response (129).

In order to determine whether Mtp vaccination stimulates a cell-mediated immune response, cytokine responses of mouse splenocytes will be determined using *ex vivo* analysis (134). Spleens from 5 mice from each study group described above will be pooled, and lymphocytes purified over a Ficoll-Hypaque gradient. CD3-positive T-cells will be enriched using a T-cell enrichment column (R & D Systems) following the procedure of Kobie *et al.* (134). Thymocytes will be re-stimulated *in vitro* using Mtp antigen or PPD pulsed bone marrow derived dendritic cells [DC] (134). These DC cultures will be prepared and matured with TNF-α as previously described (134). The DC will be primed 24 h before incubation with splenocytes by the addition of 1 or 10 μg Mtp, 1 or 10 μg of PPD (Mycos Research), or buffer alone. Controls will include DC alone and T cells alone. Supernatants will be collected after 72 h, and the levels of IFN-γ, IL-4, and IL-10 secretion will be determined by ELISA using immunoglobulin specific for mouse cytokines (PharMingen). IFN-γ cytokine production has been demonstrated to be critical in the development of a protective anti-mycobacterial immune response. Th1-type cytokines, such as IFN-γ, are critical in preventing active TB disease (122). IL-4 and IL-10 cytokine production is normally induced during a Th2- type immune response which is not optimal for control of tuberculosis infections (122, 135, 136). Thus, by monitoring the level of IFN-γ, IL-4, and IL-10 cytokine secretion in these assays we will be able to elucidate what type of cell-mediated immune response Mtp antigen induces in mice. These results will be compared to levels of cellular immunity (cytokine production) induced after TB infection of Mtp-vaccinated, BCG vaccinated, and control mice, as described above, either 7 or 14 d after aerosol challenge.

Animal studies will also be done to determine whether immunization with Mtp restricts the growth of *M. tuberculosis* in the lungs and spleens of aerosol-infected mice. Thirty days after vaccination with Mtp, BCG, or unimmunized control mice will be infected with virulent *M. tuberculosis* as described above. Numbers of viable bacilli will be determined in lungs and spleens of these mice at 4, 9, and 16 weeks after infection by plate count determinations, while histological analysis of mouse lungs will also be done as previously described. To inhibit growth of BCG from BCG-vaccinated mice and not *M. tuberculosis*, samples will be plated on 7H11-OADC agar plates containing 2-thiophenecarboxylic acid hydrazide (2 μg/ml). This compound inhibits replication of BCG while not inhibiting growth of *M. tuberculosis* (127). If Mtp vaccination is protective, lower numbers of bacteria should be recovered from the lungs and spleens of infected animals and lung pathology should be decreased as compared to the unimmunized control mice. The BCG vaccine group will be a good comparative model in these studies since it has been shown that this attenuated, live vaccine decreases mycobacterial numbers and decreases observed lung pathology in mice (127, 128).

E. HUMAN SUBJECTS.

Normal volunteers age 18-60 will be used as a source of blood. From each subject, 100 to 200 ml will be drawn from the arm vein. Donors will be reimbursed $30 for each blood donation. The blood will be used for the isolation of human blood-derived monocytes, as previously described. Donors will be observed for any complications. Complications include the possibility of bleeding from the vein puncture site, dizziness, and fainting. These are relatively rare events. The investigator will obtain consent after careful explanation of the procedures. Samples will be coded and the investigator will not have access to personal information. There is no direct benefit to donors undergoing this procedure. We believe that the information to be gained from the proposed study will be of substantial scientific value.

F. VERTEBRATE ANIMALS.

Female specific-pathogen-free C57BL/6 mice (a total of approximately 300) will be used in studies as proposed in Specific Aim No. 4. Mice will be challenged with *M. tuberculosis* via the aerosol route using a bacterial suspension that will introduce 50 to 100 bacilli into the mouse lungs over a 30 min exposure period using a Middlebrook chamber [Glas-Col, Terra Haute, IN] (127). **The aerosol chamber and tissue homogenizer for these studies will be purchased for the proposed work using funds from this grant.** This work will be done in our newly completed Biosafety Level-3 animal laboratory in the Animal Care Facility, at the University of Arizona College of Medicine. Sheldon L. Morris, Ph.D., Chief of the Laboratory of Mycobacterial Diseases and Cellular Immunology, at the Center for Biologics Evaluation and Research, FDA, in Bethesda, MD will be a collaborator for these animal studies. Please see his Biographical Sketch and attached letter of collaboration. Dr. Morris has extensive experience using this aerosol infection system to

Supplemental Material for Arizona Disease Control Research Commission Grant Proposal 9-102
Entitled: "Identification of Mtp Pili: A New Adhesin of *Mycobacterium tuberculosis.*"
PI: Richard L. Friedman, Ph.D.

1. **Initial biochemical characterization of mycobacterial pili.** Due to the difficulty encountered using traditional biochemical techniques to resolve the composition of the purified Mtp pili fibers (Preliminary Work, section 5) a more direct approach was employed involving a combination of enzymatic and chemical manipulations followed by analysis using liquid chromatography and tandem mass spectroscopy (LC-MS/MS). Our earliest attempts at dissociation of the fibers into the pilin subunits suggested that the pili resisted the various biochemical/chemical treatments. Also, due to the large molecular mass of intact fibers; the purified pili were unable to enter gel matrices during electrophoretic separation. To surmount this difficulty we have turned to our collaborators Frederick Cassels, Ph.D. at Water Reed Army Institute (see Letter of Collaboration) and Sonja Hess, Ph.D., Mass Spectrometry Facility, NIH/NIDDK, to analyze the purified pili from *M. tuberculosis* directly in solution.

Pili isolated from three different *M. tuberculosis* strains [H37Rv, CDC 1551, and H37Ra (Preliminary Work, section 3)] were examined in an effort to identify a common component present in all three pili preparations. Dilute acid hydrolysis normally hydrolyzes peptide bonds at either side of Asp and Asn to generate fragments. A small amount of purified Mtp material was placed in a sealable tube to which 1.1 µl of concentrated HCl (constant boiling quality) was added with 500 µl water. The tube was degassed and nitrogen was added to protect the sample from oxidation. The sealed tube was subjected to boiling at 108°C for 4 h, material reconstituted in 10-100 µl water, and the samples analyzed by LC-MS/MS using a QTOF2 instrument (Waters, Milford) (see Goals and Objectives No.1). Masslynx 4.0 was used to generate pkl files from mass spectral data. These pkl files were submitted to inhouse MASCOT searches allowing for 0.8 Da peptide mass tolerance and 0.2 Da fragment mass tolerance.

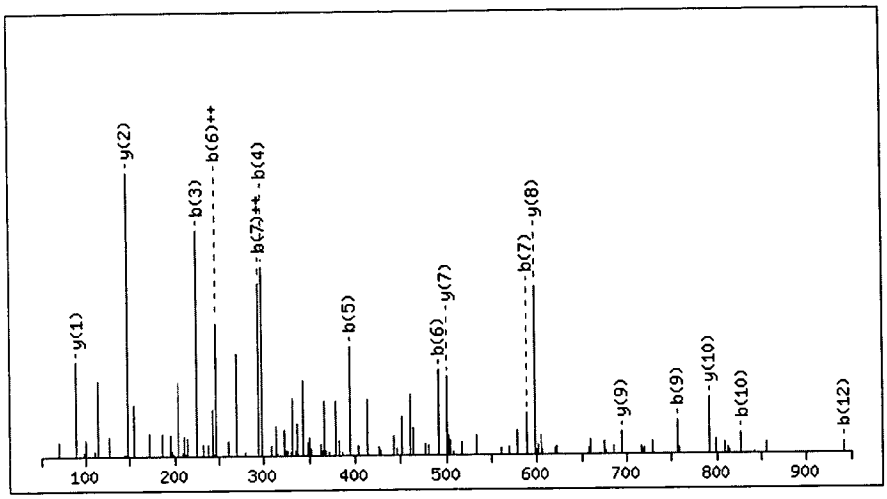
Upon examination of the LC-MS/MS results, it was found that all three samples analyzed had one common peptide fragment having a monoisotopic mass (Mr) of 1086.55 Da and a sequence of PGAAPPPPAAGGGA (aa101-114) **(Figure 1A)**. Mascot uses a probability based score and in all cases, the identification of the the fragment 101-114 was considered statistically significant, indicating a true identification. Furthermore, two of the samples produced only this peptide following acid hydrolysis indicating the relative purity of the analytes. **Obtaining identical results using three different digested Mtp samples strongly suggests that the identified fragment (Figure 1) represents a portion of the Mtp structural subunit, the MtpA pilin.**

2. **Bioinformatic analysis of the putative pilin gene.** Database BLAST searches indicated that the identified peptide sequence **(Figure 1B)** matches the predicted protein encoded by *M. tuberculosis* strain H37Rv ORF Rv3312A and strain CDC 1551 gene MT3413 **(Figure 2)**. Additionally, the identified sequence is also found in *M. bovis* ssp. *bovis* AF2212/97 as gene Mb3314c. The available genome databases of other mycobacterial strains were also examined to determine the conservation of the putative *mtpA* gene across mycobacterial species. **The pilin gene was found to be absent in the other mycobacterial genomes, indicating that the gene is restricted to members of the *M. tuberculosis* complex.** It is notable that *mtpA* is absent in *M. smegmatis* strain mc^2155 and supports our observations that the pili produced by the fast growing saprophyte are biochemically distinct from Mtp, as suggested by the morphological observations and antigenic differences reported in the preliminary data section (Preliminary Work, section 2 and 4). Outside of the indicated mycobacterial strains, the putative *mtpA* gene does not have any similarity to any prokaryotic or eukaryotic sequences deposited in the databases at NCBI. The putative *mtpA* gene is one of three conserved hyothetical proteins surrounded by intermediary metabolic genes **(Figure 3)**. Because most pili characterized belong to Gram-negative bacteria it is not surprising that the genomic organization of *mtpA* in *M. tuberculosis* H37Rv does not resemble known pili biogenesis operons.

3. **Production of antibodies against MtpA.** Antibodies will be used to determine if the identified protein from the purified pili samples is indeed the structural subunit MtpA using immunolabelling techniques. **If the identified protein sequence represents MtpA, then antibodies derived against that protein will recognize Mtp fibers in TEM-immunogold and immunofluorescence assays.** An immunogenic peptide was selected from the deduced protein sequence obtained from the Tuberculist database **(Figure 2)**. Peptides were synthesized (Zymed Laboratories) and the amino-terminal cysteine residue of the molecule was used for a single point, site-directed conjugation to KLH to increase the immunogenicity of the peptide. **The resulting hapten-peptide immunogen is presently being used to immunize rabbits and the production of antisera against the putative MtpA monomer is in progress (Zymed).** The sequence data was utilized to design PCR primers and the putative *mtpA*, with specific *Nde*I and *Xho*I restriction sites, was amplified (data not shown) for producing an in-frame N-terminal 6X His-tagged MtpA protein using the *Escherichia coli* expression vector pET15b (Novagen). Production of the His-tagged MtpA is in progress and will be useful in Western blotting experiments with the rabbit anti-MtpA antibody being produced as described above and in studies proposed in the grant application (Goals and Objectives No.1, section 3).

4. **Production of Mtp-deficient *M. tuberculosis* mutants.** The MS analysis described above provides strong suggestive evidence that the identified sequence is MtpA. Therefore, the ORF encoding the identified product has been targeted for disruption and deletion using two different techniques successfully used by our laboratory and other research groups. The first approach, already in progress, involves allelic exchange to introduce an *mtpA-hyg* allele into the chromosome using the conditionally replicating suicide vector pMJ10 to generate a *mtpA* disrupted mutant of *M. tuberculosis* (Goals and Objectives No.2, section 2). **The second approach, also in progress in our laboratory, involves a newer technique for generating *M. tuberculosis* targeted mutations using the specialized transduction system developed in the laboratory of Dr. William Jacobs, Jr. at Albert Einstein University. We have already obtained this transduction system from Dr. Jacobs.** PCR primers have been designed and utilized to amplify upstream and downstream regions of approximately 1000-bp for each product flanking the putative *mtpA* ORF (data not shown). The products will be directionally cloned into MCSs flanking the hygromycin cassette located on the cosmid pYUB854 to produce allelic exchange substrates (AES). This construct, pYUB3312, contains the upstream and downstream regions from *mtpA* in their original genome organization separated by a *hyg* cassette and will be used to generate a mutant in which the entire *mtpA* between the cloned upstream and downstream arms will be deleted from the chromosome. The *hyg* cassette also contains DNA binding sites for resolvase that will allow for unmarking the deletion, if desired. The recombinant cosmid pYUB3312 will be introduced into the conditionally-replicating phasmid vector phAE87 and the recombinant shuttle cosmids will be purified from *E. coli* transductants. The purified cosmids will be used to transfect *M. smegmatis* cells at the permissive temperature of 30°C and plaque purification will be used to recover mycobacteriophage-packaged DNA molecules. The desired mutant will be obtained by transduction of *M. tuberculosis* cells at the non-permissive temperature of 37°C to limit phage replication. Allelic exchange occurs by a double crossover event between the homologous regions surrounding the *mtpA* gene. Mutants will be selected on antibiotic containing medium to identify the desired clones.

A



B

1	98.06	49.53	P			14
2	155.08	78.04	G	990.50	495.75	13
3	226.12	113.56	A	933.48	467.24	12
4	297.16	149.08	A	862.44	431.73	11
5	394.21	197.61	P	791.41	396.21	10
6	491.26	246.13	P	694.35	347.68	9
7	588.31	294.66	P	597.30	299.15	8
8	685.37	343.19	P	500.25	250.63	7
9	756.40	378.71	A	403.19	202.10	6
10	827.44	414.22	A	332.16	166.58	5
11	884.46	442.74	G	261.12	131.06	4
12	941.48	471.25	G	204.10	102.55	3
13	998.51	499.76	G	147.08	74.04	2
14			A	90.06	45.53	1

Figure 1. Tandem mass spectroscopy fragmentation pattern of the acid hydrolysate produced from Mtp samples (A), actual identifications are indicated in red (bold) and theoretically possible fragments are black (B).

MLARSLSYRHRMYRFACRTLMLAACILATGVAGLGVGAQSAAQTAPVPDYYWCPGQPFDPA
WGPNWDPYTCHDDFHRDSDGPDHSRDYPGPILEGPVLDD**PGAAPPPPAAGGGA**-COOH

Figure 2. Predicted amino acid sequence of *M. tuberculosis* H37Rv gene Rv3312A and *M. tuberculosis* CDC1551 gene MT3413. Bold sequence indicates the peptide fragment identified by LC-MS/MS as described in the text. Shaded sequence represents the synthesized peptide used as an immunogen to generate antisera to be utilized in ongoing studies to identify the pili structural subunit.

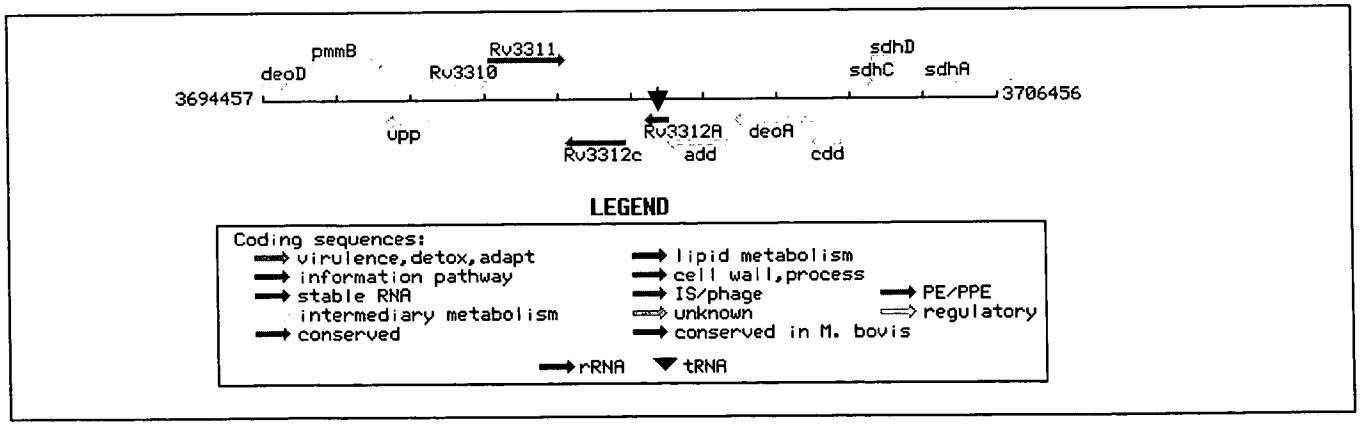


Figure 3. Genome organization of putative *mtpA* region in *M. tuberculosis* H37Rv. The arrowhead denotes the ORF coding for the identified protein as described in the text. Display obtained at: http://genolist.pasteur.fr/TubercuList/.